EP0668781

PCT

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B6

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 48/00, 35/12, 39/00
C12N 15/19, 15/24, 15/25
C12N 15/26, 15/90, 15/63

A1

(11) International Publication Number: WO 93/07906

(43) International Publication Date: 29 April 1993 (29.04.93)

(21) International Application Number: PCT/US92/08999

(22) International Filing Date: 23 October 1992 (23.10.92)

(30) Priority data:
781,356 25 October 1991 (25.10.91) US
863,641 3 April 1992 (03.04.92) US

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(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: LYMPHOKINE GENE THERAPY OF CANCER

(57) Abstract

A novel method of tumor immunotherapy is described comprising the genetic modification of cells resulting in the secretion of cytokine gene products to stimulate a patient's immune response to tumor antigens. In one embodiment, autologous fibroblasts genetically modified to secrete at least one cytokine gene product are utilized to immunize the patient in a formulation with tumor antigens at a site other than an active tumor site. In another embodiment, cells genetically modified to express at least one tumor antigen product and to secrete at least one cytokine gene product are utilized in a formulation to immunize the patient at a site other than an active tumor site.

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Lymphokine Gene Therapy of Cancer

BACKGROUND

This application is a continuation-in-part of United States Patent Application Serial No. 07/781,356, filed on October 25, 1991, which is a continuation-in-part of United States Patent Application Serial No. 07/720,872, filed on June 25, 1991, both of which are incorporated herein in their entirety.

Recent advances in our understanding of the biology of the immune system have lead to the identification of important modulators of immune responses, called cytokines (1-3). Immune system modulators produced by lymphocytes are termed lymphokines, a subset of the cytokines. These agents mediate many of the immune responses involved in anti-tumor immunity. Several of these cytokines have been produced by recombinant DNA methodology and evaluated for their anti-tumor effects. The administration of lymphokines and related immunomodulators has resulted in objective tumor responses in patients with various types of neoplasms (4-7). However, current modes of cytokine administration are frequently associated with toxicities that limit the therapeutic value of these agents.

For example, interleukin-2 (IL-2) is an important lymphokine in the generation of anti-tumor immunity (4). In response to tumor antigens, a subset of lymphocytes termed helper T-cells secrete small quantities of IL-2. This IL-2 acts locally at the site of tumor antigen stimulation to activate cytotoxic T-cells and natural killer cells which mediate systemic tumor cell destruction. Intravenous, intralymphatic and intralesional administration of IL-2 has resulted in clinically significant responses in some cancer patients (4-6). However, severe toxicities (hypotension and adema) limit the dose and efficacy of intravenous and intralymphatic IL-

2 administration (5-7). The toxicity of systemically administered lymphokines is not surprising as these agents mediate local cellular interactions and they are normally secreted in only very small quantities.

Additionally, other cytokines, such as interleukin-4 (IL-4), alpha interferon (α-INF) and gamma interferon (γ-INF) have been used to stimulate immune responses to tumor cells. Like IL-2, the current modes of administration have adverse side effects.

To circumvent the toxicity of systemic cytokine administration, several investigators have examined intralesional injection of IL-2. This approach eliminates the toxicity associated with systemic IL-2 administration (8,9,10). However, multiple intralesional injections are required to optimize therapeutic efficacy (9,10). Hence, these injections are impractical for many patients, particularly when tumor sites are not accessible for injection without potential morbidity.

An alternative approach, involving cytokine gene transfer into tumor cells, has resulted in significant anti-tumor immune responses in several animal tumor models (11-14). In these studies, the expression of cytokine gene products following cytokine gene transfer into tumor cells has abrogated the tumorigenicity of the cytokine-secreting tumor cells when implanted into syngeneic hosts. The transfer of genes for IL-2 (11,12) γ-INF (13) or interleukin-4 (IL-4) (14) significantly reduced or eliminated the growth of several different histological types of murine tumors. In the studies employing IL-2 gene transfer, the treated animals also developed systemic anti-tumor immunity and were protected against subsequent tumor challenges with the unmodified parental tumor (11,12). Similar inhibition of tumor growth and protective immunity was also demonstrated when immunizations were performed

with a mixture of unmodified parental tumor cells and genetically modified tumor cells engineered to express the IL-2 gene. No toxicity associates with localized lymphokine transgene expression was reported in these animal tumor studies (11-14).

While the above gene-transfer procedure has been shown to provide anti-tumor immunity, it still retains practical difficulties. This approach is limited by the inability to transfer functional cytokine genes into many patients' tumor cells, as most patients' tumors cannot be established to grown *in vitro* and methods for human *in vivo* gene transfer are not available.

SUMMARY OF THE INVENTION

The present invention demonstrates a novel, more practical method of cytokine cancer immunotherapy. In one approach, selected cells from a patient, such as fibroblasts, obtained, for example, from a routine skin biopsy, are genetically modified to express one or more cytokines. Alternatively, patient cells which may normally serve as antigen presenting cells in the immune system such as macrophages, monocytes, and lymphocytes may also be genetically modified to express one or more cytokines. These modified cells are hereafter called cytokine-expressing cells, ore CE cells. The CE cells are then mixed with the patient's tumor antigens, for example in the form of irradiated tumor cells, or alternatively in the form of purified natural or recombinant tumor antigen, and employed in immunizations, for example subcutaneously, to induce systemic anti-tumor immunity.

The cytokines are locally expressed at levels sufficient to induce or augment systemic anti-tumor immune responses via local immunization at sites other than active tumor sites. Systemic toxicity related to cytokine

administration should not occur because the levels of cytokine secreted by the CE cells should not significantly affect systemic cytokine concentrations.

As the amount of cytokine secreted by the CE cells is sufficient to induce anti-tumor immunity but is too low to produce substantial systemic toxicity, this approach provides the benefit of local cytokine administration. In addition, this novel method obviates the need for intralesional injections, which may produce morbidity. Furthermore, the continuous local expression of cytokine(s) at the sites of immunization may also augment anti-tumor immune responses compared to intermittent cytokine injections. This method also provides the advantage of local immunization with the CE cells, as opposed to cumbersome intravenous infusions. This method also eliminates the need for establishing tumor cell lines in vitro as well as transfer of genes into these tumor cells.

This invention also provides an alternative means of localized expression of cytokines to induce and/or increase immune responses to a patient's tumor through genetic modification of cellular expression of both cytokine(s) and tumor antigen(s). In this embodiment, selected cells from a patient are isolated and transduced with cytokine gene(s) as well as gene(s) coding for tumor antigen(s). The transduced cells are called "carrier cells." Carrier cells can include fibroblasts and cells which may normally serve as antigen presenting cells in the immune system such as macrophages, monocytes, and lymphocytes. Transduced carrier cells actively expressing both the cytokine(s) and the tumor antigen(s) are selected and utilized in local immunizations at a site other than active tumor sites to induce anti-tumor immune responses. As with the CE cells, these carrier cells should not produce substantial systemic toxicities, as the levels of

cytokine(s) secreted by the carrier cells should not significantly affect systemic cytokine concentrations. This alternate embodiment is advantageous because it obviates the need to obtain samples of the tumor, which is sometimes difficult. However, carrier cells can be utilized in local immunizations in conjunction with tumor cells, tumor cell homogenates, purified tumor antigens, or recombinant tumor antigens to enhance anti-tumor immunity.

Additionally, this second embodiment retains the same advantages as the first embodiment in that the level of cytokine released by the carrier cells is sufficient to induce anti-tumor immunity but is too low to produce substantial systemic toxicity. In addition, as with the first embodiment, this method obviates the need for intralesional injections, and allows for continuous expression of cytokine(s). This method also eliminates the need for establishing continuous cultures in vitro of tumor cells as well as transfer of genes into these tumor cells, and provides the advantage of local immunization with the carrier cells, as opposed to cumbersome lengthy intravenous infusions.

These approaches may also find application in inducing or augmenting immune responses to other antigens of clinical significance in other areas of medical practice.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows schematic diagrams of retroviral vectors DC/TKIL2, LXSN-IL2, and LNCX-IL2.

Figure 2 shows a mean IL-2 concentration of triplicate supernatant samples measured by ELISA. Supernatants were harvested from overnight cultures of approximately 1.5×10^6 semi-confluent fibroblasts.

Figure 3 shows biological activity of the IL-2 secreted by the transduced fibroblasts was demonstrated by measuring mean ^{3}H-TdR incorporation of an IL-2 dependent T-cell line incubated with triplicate samples of supernatants. Supernatants were harvested from overnight cultures of approximately 1.5 x 10^{6} semi-confluent fibroblasts.

Figure 4 shows comparisons between animals injected with 10^{5} CT26 tumor cells alone (□); 10^{5} CT26 tumor cells and 2 x 10^{6} unmodified BALB/C fibroblasts (■); 10^{5} CT26 tumor cells and 2 x 10^{6} IL-2 transduced BALB/C fibroblasts (●); and 10^{5} CT26 tumor cells and 1 x 10^{6} transduced BALB/C fibroblasts (○). Tumor measurements are the mean products of the cross-sectional diameter of the tumors from four animals in each treatment group. The (*) indicates statistically significant difference ($P < 0.05$) in tumor growth curves.

Figure 5 shows PCR analysis of neomycin phosphotransferase DNA sequences. Lane 1 - positive control pLXSN-RI-IL2. Lanes 2 through 4 tests genomic DNA; Lanes 5 and 6 ovary genomic DNA; Lane 7 negative control, no DNA. Identical results were obtained with liver, spleen and lung genomic DNA (data not shown).

Figure 6 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2 x 10^{6} fibroblasts mixed with 5 x 10^{4} CT26 tumor cells concentrating on the rate of tumor growth.

Figure 7 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2 x 10^{6} fibroblasts mixed with 5 x 10^{4} CT26 tumor cells concentrating on the time of tumor onset for the individual animal in each treatment group.

Figure 8 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2 x 10^6 fibroblasts mixed with 1 x 10^5 CT26 tumor cells concentrating on the rate of tumor growth.

Figure 9 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2 x 10^6 fibroblasts mixed with 1 x 10^5 CT26 tumor cells concentrating on the time of tumor onset for the individual animal in each treatment group.

Figure 10 shows the effect of IL-2 modified cells on tumor establishment and development using 2 x 10^6 DCTK-IL2-modified CT26 tumor cells mixed with 1 x 10^5 unmodified CT26 compared to 2 x 10^6 DCTK-IL2-modified fibroblasts mixed with 1 x 10^5 CT26 concentrating on the rate of tumor growth.

Figure 11 shows the effect of IL-2 modified cells on tumor establishment and development using 2 x 10^6 DCTK-IL2-modified CT26 tumor cells mixed with 1 x 10^5 unmodified CT26 compared to 2 x 10^6 DCTK-IL2-modified fibroblasts mixed with 1 x 10^5 CT26 concentrating on the time of tumor onset for the individual animal in each treatment group.

Figure 12 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the rate of tumor growth. Mice were immunized with 2 x 10^6 fibroblasts mixed with 2.5 x 10^5 irradiated CT26 tumor cells 7 days prior to challenge with 5 x 10^4 fresh tumor cells.

Figure 13 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the time of tumor onset for the individual animal in each treatment group. Mice were immunized with 2 x 10^6 fibroblasts mixed with 2.5 x 10^5 irradiated CT26 tumor cells 7 days prior to challenge with 5 x 10^4 fresh tumor cells.

Figure 14 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the rate of tumor growth. Mice were immunized with 2 x 10^6 fibroblasts mixed with 2.5 x 10^5 irradiated CT26 tumor cells 14 days prior to challenge with 5 x 10^4 fresh tumor cells.

Figure 15 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the time of tumor onset for the individual animal in each treatment group. Mice were immunized with 2 x 10^6 fibroblasts mixed with 2.5 x 10^5 irradiated CT26 tumor cells 14 days prior to challenge with 5 x 10^4 fresh tumor cells.

DETAILED DESCRIPTION

A novel method of tumor immunotherapy is described comprising the genetic modification of cells resulting in the secretion of cytokine gene products to stimulate a patient's immune response to tumor antigens. "Gene" is defined herein to be a nucleotide sequence encoding the desired protein. In one embodiment, autologous fibroblasts genetically modified to secrete at least one cytokine gene product are utilized to immunize the patient in a formulation with tumor antigens at a site other than an active tumor site. In another embodiment, cells genetically modified to express at least one tumor antigen gene product and to secrete at least one cytokine gene product are utilized in formulation to immunize the patient at a site other than an active tumor site. Cytokines are preferably expressed in cells which efficiently secrete these proteins into the surrounding milieu. fibroblasts are an example of such cells. Fibroblasts or other cells can be genetically modified to express and secrete one or more cytokines, as described later in this specification.

Tumor antigens can be provided by several methods, including, but not limited to the following: 1) CE cells can be transduced with gene(s) coding for tumor antigens. These "carrier cells" are then utilized in patient immunizations. 2) Cloned gene sequences coding for appropriate tumor antigens can be transferred into cells such as fibroblasts or antigen-presenting cells. These cells are then mixed with CE or carrier cells to immunize the patient. 3) Tumor antigens can be cloned in bacteria or other types of cells by recombinant procudures. These antigens are then purified and employed an immunization with CE and/or carrier cells. 4) Tumor antigens can be purified from tumor cells and used, along with CE or carrier cells, to immunize the patient. 5) Tumor cells may be irradiated or mechanically disrupted and mixed with CE and/or carrier cells for patient immunizations.

This invention encompasses the following steps: (A) isolation of appropriate cells for generation of CE cells or carrier cells; (B) isolation of cytokine genes or isolation of cytokine genes and tumor antigen genes, as well as appropriate marker and/or suicide genes; (C) transfer of the genes from (B) to produce the CE cells or carrier cells; (D) preparation of immunological samples of the patient's tumor antigens or other suitable tumor antigens for immunization with CE or carrier cells; (E) inactivation of the malignant potential of tumor cells if they are used as a source of tumor antigens for immunization; and (F) preparation of samples for immunization. Following are several embodiments contemplated by the inventors. However, it is understood that any means known by those in the art to accomplish these steps will be usable in this invention.

(A) Isolation of Cells to Generate CE and Carrier Cells

Cells to be utilized as CE cells and carrier cells can be selected from a variety of locations in the patient's body. For example, skin punch biopsies provide a readily available source of fibroblasts for use in generating CE cells, with a minimal amount of intrusion to the patient. alternatively, these fibroblasts can be obtained from the tumor sample itself. Cells of hematopoietic origin may be obtained by venipuncture, bone marrow aspiration, lymph node biopsies, or from tumor samples. Other appropriate cells for the generation of CE or carrier cells can be isolated by means known in the art. Non-autologous cells similarly selected and processed can also be used.

(B) Isolation of Genes

Numerous cytokine genes have been cloned and are available for use in this protocol. The genes for IL-2, γ-INF and other cytokines are readily available (1-5, 11-14). Cloned genes of the appropriate tumor antigens are isolated according to means known in the art.

Selectable marker genes such as neomycin resistance (Neo^R) are readily available. Incorporation of a selectable marker gene(s) allows for the selection of cells that have successfully received and express the desired genes. Other selectable markers known to those in the art of gene transfer may also be utilized to generate CE cells or carrier cells expressing the desired transgenes.

"Suicide" genes can be incorporated into the CE cells or carrier cells to allow for selective inducible killing after stimulation of the immune response. A gene

such as the herpes simplex virus thymidine kinase gene (TK) can be used to create an inducible destruction of the CE cells or carrier cells. When the CE cells or carrier cells are no longer useful, a drug such as acyclovir or gancyclovir can be administered. Either of these drugs will selectively kill cells expressing TK, thus eliminating the implanted transduced cells. Additionally, a suicide gene may be a gene coding for a non-secreted cytotoxic polypeptide attached to an inducible promoter. When destruction of the CE or carrier cells is desired, the appropriate inducer of the promoter is administered so that the suicide gene is induced to produce cytotoxic polypeptide which subsequently kills the CE or carrier cell. However, destruction of the CE or carrier cells may not be required.

Genes coding for tumor antigen(s) of interest can be cloned by recombinant methods. The coding sequence of an antigen expressed by multiple tumors may be utilized for many individual patients.

(C) Transfer of Genes

Numerous methods are available for transferring genes into cultured cells (15). For example, the appropriate genes can be inserted into vectors such as plasmids or retroviruses and transferred into the cells. Electroporation, lipofection and a variety of other methods are known in the field and can be implemented.

One method for gene transfer is a method similar to that employed in previous human gene transfer studies, where tumor infiltrating lymphocytes (TILs) were modified by retroviral gene transduction and administered to cancer patients (16). In this Phase I safety study of retroviral mediated gene transfer, TILs were genetically modified to express the Neomycin resistance (Neo^R) gene. Following

intravenous infusion, polymerase chain reaction analyses consistently found genetically modified cells in the circulation for as long as two months after administration. No infectious retroviruses were identified in these patients and no side effects due to gene transfer were noted in any patients (16). These retroviral vectors have been altered to prevent viral replication by the deletion of viral gag, pol and env genes.

When retroviruses are used for gene transfer, replication competent retroviruses may theoretically develop by recombination between the retroviral vector and viral gene sequences in the packaging cell line utilized to produce the retroviral vector. We will use packaging cell lines in which the production of replication competent virus by recombination has been reduced or eliminated. Hence, all retroviral vector supernatants used to infect patient cells will be screened for replication competent virus by standard assays such as PCR and reverse transcriptase assays (16). Furthermore, exposure to replication competent virus may not be harmful. In studies of subhuman primates injected with a large inoculum of replication competent murine retrovirus, the retrovirus was cleared by the primate immune system (17). No clinical illnesses or sequelae resulting from replication competent virus have been observed three years after exposure. In summary, it is not expected that patients will be exposed to replication competent murine retrovirus and it appears that such exposure may not be deleterious (17).

(D) <u>Preparation of Immunological Samples of the Patient's Tumor Antigens or Purified Recombinant Tumor Antigens</u>

Tumor cells bearing tumor associated antigens are isolated from the patient. These cells can derive either from solid tumors or from leukemic tumors. For solid

tumors, single-cell suspensions can be made by mechanical separation and washing of biopsy tissue (18).

Hematopoietic tumors may be isolated from peripheral blood or bone marrow by standard methods (19).

A second variant is the use of homogenates of tumor cells. Such homogenates would contain tumor antigens available for recognition by the patient's immune system upon stimulation by this invention. Either unfractionated cell homogenates, made, for example, by mechanical disruption or by freezing and thawing the cells, or fractions of homogenates preferably with concentrated levels of tumor antigens, can be used.

Likewise, purified tumor antigens, obtained for example by immunoprecipitation or recombinant DNA methods, could be used. Purified antigens would then be utilized for immunizations together with the CE cells and/or carrier cells described above to induce or enhance the patient's immune response to these antigens.

In the embodiments employing carrier cells, tumor antigens are available through their expression by the carrier cells. These carrier cells can be injected alone or in conjunction with other tumor antigen preparations or CE cells. Likewise, when CE cells are used, purified recombinant tumor antigen, produced by methods known in the art, can be used.

If autologous tumor cells are not readily available, heterologous tumor cells, their homogenates, their purified antigens, or carrier cells expressing such antigens could be used.

(E) Inactivation of Tumor Cells

When viable tumor cells are utilized in immunizations as a source of tumor antigens, the tumor cells can be inactivated so that they do not grow in the patient. Inactivation can be accomplished by several methods. the cells can be irradiated prior to immunization (18). This irradiation will be at a level which will prevent their replication. Such viable calls can then present their tumor antigens to the patient's immune system, but cannot multiply to create new tumors.

Alternatively, tumor cells that can be cultured may be transduced with a suicide gene. As described above, a gene such as the herpes simplex thymidine kinase (TK) gene can be transferred into tumor cells to induce their destruction by administration of acyclovir or gancyclovir. After immunization, the TK expressing tumor cells can present their tumor antigens, and are capable of proliferation. After a period of time during which the patients's immune response is stimulated, the cells can be selectively killed. This approach might allow longer viability of the tumor cells utilized for immunizations, which may be advantageous in the induction or augmentation of anti-tumor immunity.

(F) Preparation of Samples for Immunization

CE cells and/or carrier cells and tumor cells, and/or homogenates of tumor cells and/or purified tumor antigen(s), are combined for patient immunization. Approximately 10^7 tumor cells will be required. If homogenates of tumor cells or purified or non-purified fractions of tumor antigens are used, the tumor dose can be adjusted based on the normal number of tumor antigens usually present on 10^7 intact tumor cells. The tumor preparation should be mixed with numbers of CE or carrier

cells sufficient to secrete cytokine levels that induce anti-tumor immunity (11-12) without producing substantial systemic toxicity which would interfere with therapy.

The cytokines should be produced by the CE cells or the carrier cells at levels sufficient to induce or augment immune response but low enough to avoid substantial systemic toxicity. This prevents side effects created by previous methods' administration of greater than physiological levels of the cytokines.

These mixtures, as well as carrier cells that are utilized alone, will be formulated for injection in any manner known in the art acceptable for immunization. Because it is important that at least the CE cells and carrier cells remain viable, the formulations must be compatible with cell survival. Formulations can be injected subcutaneously, intramuscularly, or in any manner acceptable for immunization.

Contaminants in the preparation which may focus the immune response on undesired antigens should be removed prior to the immunizations.

The following examples are provided for illustration of several embodiments of the invention and should not be interpreted as limiting the scope of the invention.

EXAMPLE I

IMMUNIZATION WITH FIBROBLASTS EXPRESSING IL-2 MIXED WITH IRRADIATED TUMOR CELLS

1) Isolation of Autologous Fibroblasts for Use in Generating IL-2 Secreting CE Cells

Skin punch biopsies will be obtained from each patient under sterile conditions. The biopsy tissue will be minced and placed in RPMI 1640 media containing 10% fetal calf serum (or similar media) to establish growth of the skin fibroblasts in culture. The cultured fibroblasts will be utilized to generate IL-2 secreting CE cells by retroviral mediated IL-2 gene transfer.

2) Retroviral Vector Preparation and Generation of IL-2 Secreting CE Cells

The cultured skin fibroblasts will then be infected with a retroviral vector containing the IL-2 and Neomycin resistance (Neo^R) genes. An N2 vector containing the Neo^R gene will be used, and has been previously utilized by a number of investigators for in vitro and in vivo work, including investigations with human subjects (16). The IL-2 vector will be generated from an N2-derived vector, LLRNL, developed and described by Friedmann and his colleagues (20). It will be made by replacement of the luciferase gene of LLRNL with a full-length cDNA encoding human IL-2. Retroviral vector free of contaminating replication-competent virus is produced by transfection of vector plasmid constructions into the helper-free packaging cell line PA317. Before infection of patients' cells, the vector will have been shown to be free of helper virus. In the event that helper virus is detected, the vector will be produced in the GP + envAM12 packaging cell line in which

the viral gag and pol genes are separated from the env, further reducing the likelihood of helper virus production.

3) Transduction Protocol

The cultured primary fibroblasts will be incubated with supernatant from the packaging cell line as described (20). Supernatant from these cells will be tested for adventitious agents and replication competent virus as described (16) and outlined in Table 1. The fibroblasts are washed and then grown in culture media containing G418, (a neomycin analogue) to select for transduced cells expressing the Neo^R gene. The G418-resistant cells will be tested for expression of the IL-2 gene by measuring the concentration of IL-2 in the culture supernatant by an enzyme linked immunosorbent assay (ELISA) (12). G418-resilient cells expressing IL-2 will be stored at -70°C until required for subsequent use in immunizations.

Table 1

Adventitious Agents and Safety Testing

1. Sterility
2. Mycoplasma
3. General Safety
4. Viral Testing
   LCM Virus
   Thymic agent
   S+/L- eco
   S+/L-xeno
   S+/L- ampho
   3T3 amplification
   MRC-5/Vero

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4) Preparation of Irradiated Tumor Cells

Tumors obtained form clinically indicated surgical resections or from superficial lymph node or skin metastases will be minced into 2-3 mm pieces and treated with collagenase and DNAse to facilitate separation of the tumor into a single cell suspension. The collected cells will be centrifuged and washed in RPMI 1640 media and then cryopreserved in a solution containing 10% dimethyl sulphoxide and 50% fetal calf serum in RPMI 1640 media. The cells will be stored in liquid nitrogen until the time of administration. Prior to their use in subcutaneous immunizations, the cells will be thawed, washed in media free of immunogenic contaminants, and irradiated with 4,000 rads per minute for a total of 20,000 rads in a cesium irradiator.

5) Patient Selection

Patients will have a histologically confirmed diagnosis of cancer. Patients with tumors that must be resected for therapeutic purposes or with tumors readily accessible for biopsy are most appropriate for this embodiment of the invention.

6) Pretreatment Evaluation

The following pretreatment evaluations will be performed:

1) History and physical examination including a description and quantification of disease activity.

2) Performance Status Assessment

0 = Normal, no symptoms
1 = Restricted, but ambulatory
2 = Up greater than 50% of waking hours, capable of self-care
3 = Greater than 50% of waking hours confined to bed or chair, limited self-care
4 = Bedridden

3) Pretreatment Laboratory:

CBC with differential, platelet count, PT, PTT, glucose, BUN, creatinine, electrolytes, SGOT, SGPT, LDH, alkaline phosphatase, bilirubin, uric acid, calcium, total protein albumin.

4) Other Analyses:

Urinalysis

CH_{50}, C_3 and C_4 serum complement levels

Immunophenotyping of peripheral blood B cell and T cell subsets

Assays for detectable replication-competent virus in peripheral blood cells

PCR assays of peripheral blood leukocytes for Neo^R, IL-2 and viral env

5) Other Pretreatment Evaluation:

Chest X-ray and other diagnostic studies including computerized tomography (CT), magnetic resonance imaging (MRI) or radionuclide scans may be performed to document and quantify the extent of disease activity.

Follow-up evaluations of these assessments at regular intervals during the course of therapy (approximately every 1 to 3 months) will be useful in determining response to therapy and potential toxicity.

permitting adjustments in the number of immunizations administered.

7) Restrictions on Concurrent Therapy

For optimal effects of this treatment, patients should receive no concurrent therapy which is known to suppress the immune system.

8) Final Formulation

Each patient will receive subcutaneous immunizations with a mixture if irradiated tumor cells and autologous fibroblast CE cells genetically modified to secrete IL-2. Approximately 10^7 tumor cells will be mixed with 10^7 fibroblasts known to secrete at least 20 units/ml of IL-2 in tissue culture when semi-confluent (12). The irradiated tumor cells and genetically modified fibroblasts will be placed in a final volume of 0.2 ml normal saline for immunization.

9) Dose Adjustments

At least two subcutaneous immunizations will be administered, two weeks apart, with irradiated tumor cells and autologous fibroblasts genetically modified to secrete IL-2. If no toxicity is observed, subsequent booster immunizations may be administered periodically (at least one week apart) to optimize the anti-tumor immune response.

J) Treatment of Potential Toxicity

Toxic side effects are not expected to result from these immunizations. However, potential side effects of these immunizations are treatable in the following manner:

If massive tumor cell lysis results, any resulting uric acid nephropathy, adult respiratory distress syndrome, disseminated intravascular coagulation or hyperkalemia will be treated using standard methods.

Local toxicity at the sites of immunization will be treated with either topical steroids and/or surgical excision of the injection site as deemed appropriate.

Hypersensitivity reactions such as chills, fever and/or rash will be treated symptomatically with antipyretics and antihistamines. Patients should not be treated prophylactically. Should arthralgias, lymphadenopathy or renal dysfunction occur, treatment with corticosteroids and/or antihistamines will be instituted. Anaphylaxis will be treated by standard means such as administration of epinephrine, fluids, and steroids.

EXAMPLE II

A. Retroviral IL-2 Gene Transfer and Expression in Fibroblasts

Retroviral vectors were employed to transfer and express IL-2 and neomycin phosphotransferase genes in murine and primary human fibroblasts. The retroviral vector DC/TKIL2 produced by Gilboa and co-workers (Gansbacher, et al., J. Exp. Med. 172:1217-1223, 1990, which is incorporated herein by reference) was utilized to transduce murine fibroblasts for application in an animal tumor model (see Section B below). Human fibroblasts were transduced with the retroviral vector LXSN-RI-IL2. Schematic diagrams of the structure of these retroviral vectors are provided in Figure 1. A more complete description of the LXSN-RI-IL2 vector, including its nucleotide sequence, is provided in Example III and in Tables 2, 3 and 4.

Following infection with the described vectors and selection for 2-3 weeks in growth media containing the neomycin analogue G418, Balb/c and human embryonic fibroblast culture supernatants were harvested and tested for IL-2 by an enzyme-linked immunosorbent assay (ELISA). Figure 2 depicts the levels of IL-2 secreted by the transduced fibroblasts.

These results can be confirmed using negative control fibroblasts infected with an N2-derived retroviral vector expressing an irrelevant gene such as luciferase or ß-galactosidase and studies with adult human fibroblasts.

Biological activity of the IL-2 expressed by the transduced human fibroblasts was confirmed by a cell proliferation bioassay employing an IL-2 dependent T cell line. In this assay, supernatant from the transduced fibroblasts and control unmodified fibroblasts were incubated with the IL-2 dependent T cell line CTLL-2. Incorporation of ^{3}H-thymidine was measured as an indicator of cell proliferation and IL-2 activity (Figure 3).

B. <u>Efficacy of Transduced Fibroblasts in an Animal Tumor Model</u>

The efficacy of fibroblasts genetically modified to secrete IL-2 was tested in an animal model of colorectal carcinoma. In these studies, the Balb/c CT26 tumor cell line was injected subcutaneously with Balb/c fibroblasts transduced to express IL-2. Control groups included animals injected with 1) a mixture of CT26 tumor cells and unmodified fibroblasts; 2) CT26 tumor cells without fibroblasts and 3) transduced fibroblasts alone. No tumors were detected in 3/8 animals treated with transduced fibroblasts and CT26 cells. In contrast, all untreated control animals (8/8) injected with CT26 tumor cells developed palpable tumors. No tumors were detected in the

animals inoculated with transduced fibroblasts without CT26 tumor cells. The mean CT26 tumor size in Balb/c mice injected with the IL-2 secreting fibroblasts was considerably smaller compared to the control groups (Figure 4). A multivariate non-parametric statistical procedure (Koziol, et al., Biometries 37:383-390, 1981 and Koziol, et al., Computer Prog. Biomed. 19:69-74, 1984, which is incorporated herein by reference) was utilized to evaluate differences in tumor growth among the treatment groups. The tumor growth curves for the four treatment groups presented in Figure 4 were significantly different ($p=0.048$). Subsequent comparisons between treatment groups revealed a significant difference ($p < 0.05$) in tumor growth between animals injected with CT26 tumor cells alone and animals treated with 2×10^6 transduced fibroblasts and CT26 tumor cells (Figure 4).

EXAMPLE III

A. Project Overview

Lymphokine gene therapy of cancer will be evaluated in cancer patients who have failed conventional therapy. An N2-derived vector containing the neomycin phosphotransferase gene will be used. This vector has been employed by a number of investigators for *in vitro* and *in vivo* studies including recently approved investigations with human subjects (Rosenberg et al., N. Eng. J. Med., 323:570-578, 1990). The lymphokine vectors used in this investigation will be generated from the N2-derived vector, LXSN, developed and described by Miller et al., Mol. Cell Biol. 6:2895, 1986 and Miller et al., BioTechniques 7:980, 1989, which are incorporated herein by reference. The vector LXSN-RI-IL2 contains human IL-2 cDNA under the control of the retroviral 5′ LTR promoter and the neomycin phosphotransferase gene under the control of the SV40 promoter (see Figure 1). The normal human IL-2 leader

sequence has been replaced with a chimeric sequence containing rat insulin and human IL-2 leader sequences (see Tables 2, 3 and 4). This chimeric leader sequence enhances IL-2 gene expression.

To construct the LXSN-RI-IL2 vector, the bacterial plasmid pBC12/CMV/IL2 (Cullen, B.R., DNA 7:645-650, 1988, which is incorporated herein by reference) containing the full-length IL-2 cDNA and chimeric leader sequence was digested with HindIII and the ends were blunted using Klenow polymerase. IL-2 cDNA was subsequently released from the plasmid by digestion with BamHI. The IL-2 fragment was purified by electrophoresis in a 1% agarose gel and the appropriate band was extracted utilizing a glass powder method. Briefly, the gel slice was dissolved in 4M NaI at 55°. After cooling to room temperature, 4 μl of oxidized silica solution (BIO-101, La Jolla, CA) was added to adsorb the DNA. The silica was ythen washed with a cold solution of 50% ethanol containing 0.1 M NaCl in TE buffer. The DNA was eluted from the silica by heating at 55° in distilled H_2O. The purified IL-2 cDNA was then directionally ligated into the HpaI-BamHI cloning sites of the pLXSN vector. A more complete description of the pLXSN-RI-IL2 vector and its partial nucleotide sequence are provided in Tables 2, 3, 4, 5 and 6.

Table 2

Description of the LXSN-RI-IL2 from position 1 to 6365

Bases	Description
1-589	Moloney murine sarcoma virus 5' LTR
659-1458	The sequence of the extended packaging signal
1469-2151	IL-2 cDNA with chimeric leader sequence
1469-1718	IL-2 chimeric leader sequence
1647-1718	coding region of the signal peptide
1719-2151	Mature IL-2 coding sequence
2158-2159	Mo mu sarcoma virus end/SV 40 start
2159-2503	Simian virus 40 early promoter
2521-2522	Simian virus DNA end/Tn5 DNA start
2557-3351	Neomycin phosphotransferase
3370-3371	Tn5 DNA end/Moloney murine leukemia virus start
3411-4004	Moloney murine leukemia virus 3' LTR
4073-4074	Moloney murine leukemia DNA end/pBR322 DNA start
4074-6365	Plasmid backbone

Table 3

Enzyme	[# Cuts]	Position(s)
Aat1	[2]	1961, 2481
Aat2	[2]	811, 6295
Acc1	[1]	4252
Acc2	[19]	392, 394, 445, 969, 971, 1193, 2751, 3052, 3084, 3807, 3809, 4081, 4083, 4186, 4527, 5108, 5438, 5931, 6263
Acy1	[5]	808, 2685, 3860, 5910, 6292
Afl1	[13]	260, 273, 328, 626, 756, 1277, 3201, 3676, 3689, 3744, 4041, 5511, 5733
Afl2	[4]	34, 1064, 1955, 3446
Afl3	[2]	1592, 4480
Aha1	[20]	161, 237, 473, 474, 602, 644, 789, 2689, 2849, 3578, 3653, 3888, 3889, 4017, 4059, 4126, 4161, 4860, 5556, 5907
Aha2	[5]	808, 2685, 3860, 5910, 6292
Aha3	[3]	5239, 5258, 5950
Alu1	[33]	29, 33, 119, 190, 411, 654, 734, 742, 1470, 1486, 1751, 1935, 2003, 2446, 2500, 2791, 3249, 3441, 3445, 3532, 3607, 3826, 4069, 4122, 4141, 4422, 4648, 4738, 4784, 5041, 5562, 5662, 5725
Alw1	[20]	1110, 1414, 1665, 2018, 2147, 2160, 2529, 2553, 2864, 2929, 3110, 4027, 5041, 5127, 5129, 5225, 5226, 5689, 6006, 6010
AlwN1	[4]	231, 3572, 3647, 4896
Aoc1	[2]	847, 1076
Aoc2	[19]	323, 413, 426, 597, 1583, 1721, 2631, 2724, 2798, 2988, 3050, 3739, 3828, 3841, 4012, 4300, 4798, 5959, 6044
Aos1	[2]	2787, 5595
ApaL1	[4]	1717, 4296, 4794, 6040

Apy1	[22]	315, 623, 801, 814, 1227, 1252, 1275, 1295, 1325, 1526, 1536, 1558, 1630, 2196, 2251, 2268, 3072, 3731, 4038, 4508, 4629, 4642
Aqu1	[6]	241, 472, 1998, 3821, 3854, 3887
Ase1	[2]	1801, 5545
Asp700	[1]	5972
Asp718	[2]	476, 3891
AspA1	[1]	1145
Asu1	[29]	169, 200, 245, 260, 273, 328, 626, 756, 826, 839, 1043, 1254, 1277, 1532, 1649, 3201, 3541, 3586, 3616, 3661, 3676, 3689, 3744, 4041, 5415, 5494, 5511, 5733, 6349
Ava1	[6]	241, 472, 1998, 3821, 3854, 3887
Ava2	[13]	260, 273, 328, 626, 756, 1277, 3201, 3676, 3689, 3744, 4041, 5511, 5733
Ava3	[2]	2232, 2304
Avr2	[2]	1962, 2482
Bal1	[3]	658, 1169, 2767
BamH1	[1]	2152
Ban1	[9]	318, 476, 1200, 2684, 2719, 3734, 3859, 3891, 5321
Ban2	[8]	413, 426, 597, 1583, 3050, 3828, 3841, 4012
Bbe1	[2]	2688, 3863
Bbv1	[22]	969, 997, 1738, 2493, 2632, 2758, 2800, 2816, 2909, 3321, 4060, 4131, 4228, 4372, 4390, 4809, 4899, 4902, 5108, 5411, 5600, 5802
Bcl1	[1]	2526
Bgl1	[2]	2435, 5493
Bsp1286I	[19]	323, 413, 426, 597, 1583, 1721, 2631, 2724, 2798, 2988, 3050, 3739, 3828, 3841, 4012, 4300, 4798, 5959, 6044

BspH1	[3]	5200, 6208, 6313
BspM1	[4]	1501, 2500, 2572, 2953
BssH2	[4]	392, 443, 3082, 3807
BstE2	[1]	1145
BstN1	[22]	315, 623, 801, 814, 1227, 1252, 1275, 1295, 1325, 1526, 1536, 1558, 1630, 2196, 2251, 2268, 3072, 3731, 4038, 4508, 4629, 4642
BstU1	[19]	392, 394, 445, 969, 971, 1193, 2751, 3052, 3084, 3807, 3809, 4081, 4083, 4186, 4527, 5108, 5438, 5931, 6263
BstX1	[1]	2060
BstY1	[11]	2010, 2152, 2521, 2856, 3102, 5121, 5132, 5218, 5230, 5998, 6015
Bsu36I	[2]	847, 1076
Ccr1	[1]	1998
Cfo1	[31]	394, 396, 445, 447, 714, 971, 2679, 2687, 2751, 2788, 3054, 3084, 3086, 3314, 3809, 3811, 3862, 4083, 4186, 4216, 4357, 4390, 4660, 4727, 4827, 5001, 5110, 5503, 5596, 5933, 6265
Cfr1	[9]	656, 790, 1167, 1188, 2591, 2765, 3156, 3183, 5761
Cfr10I	[3]	3004, 3185, 5453
Cfr13I	[29]	169, 200, 245, 260, 273, 328, 626, 756, 826, 839, 1043, 1254, 1277, 1532, 1649, 3201, 3541, 3586, 3616, 3661, 3676, 3689, 3744, 4041, 5415, 5494, 5511, 5733, 6349
Cvn1	[2]	847, 1076
Dde1	[23]	75, 165, 191, 282, 553, 847, 1076, 1348, 1692, 2442, 3348, 3487, 3582, 3657, 3698, 3879, 3967, 4290, 4755, 5164, 5330, 5870, 6296
Dpn1	[30]	95, 1104, 1236, 1421, 1659, 2012, 2154, 2523, 2528, 2547, 2858, 2936, 3017, 3026, 3104, 3507, 4021, 5048, 5123, 5134, 5142, 5220, 5232, 5337, 5678, 5696, 5742, 6000, 6017, 6053

Dra1 [3] 5239, 5258, 5950

Dra2 [4] 328, 1277, 3744, 6349

Eae1 [9] 656, 790, 1167, 1188, 2591, 2765,
3156, 3183, 5761

Eag1 [2] 790, 2591

Eco47I [13] 260, 273, 328, 626, 756, 1277,
3201, 3676, 3689, 3744, 4041, 5511, 5733

Eco52I [2] 790, 2591

Eco81I [2] 847, 1076

EcoN1 [2] 850, 1450

EcoO109I [4] 328, 1277, 3744, 6349

EcoR1 [1] 1460

EcoR1* [14] 938, 1037, 1460, 1798, 1805, 1928,
2064, 2121, 2236, 2308, 2400, 5240, 5546,
5801

EcoR2 [22] 313, 621, 799, 812, 1225, 1250,
1273, 1293, 1323, 1524, 1534, 1556, 1628,
2194, 2249, 2266, 3070, 3729, 4036, 4506,
4627, 4640

EcoR5 [4] 137, 213, 3554, 3629

EcoT22I [2] 2232, 2304

Fdi2 [2] 2787, 5595

Fnu4H1 [41] 793, 967, 983, 986, 1191, 1752,
2430, 2507, 2594, 2646, 2657, 2747, 2752,
2789, 2830, 2917, 2920, 2923, 3159, 3255,
3296, 3310, 4074, 4120, 4217, 4270, 4386,
4404, 4407, 4525, 4680, 4823, 4888, 4891,
5097, 5425, 5614, 5764, 5791, 5886, 6115

FnuD2 [19] 392, 394, 445, 969, 971, 1193,
2751, 3052, 3084, 3807, 3809, 4081, 4083,
4186, 4527, 5108, 5438, 5931, 6263

Fok1 [13] 498, 1198, 1358, 1679, 2333, 2552,
3009, 3034, 3912, 4168, 5339, 5520, 5807

Fsp1 [2] 2787, 5595

Hae2 [4] 2688, 3863, 4358, 4728

```
Hae3      [   35]   171,   202,   247,   658,   792,   828,
   840,  1045,  1169,  1190,  1255,  1534,  1650,
  1866,  1961,  2423,  2429,  2438,  2481,  2593,
  2767,  3158,  3185,  3543,  3588,  3618,  3663,
  4495,  4506,  4524,  4958,  5416,  5496,  5763,
  6350

Hap2      [   30]   161,   237,   473,   601,   643,   789,
  2590,  2667,  2689,  2717,  2848,  2938,  3005,
  3186,  3578,  3653,  3888,  4016,  4058,  4126,
  4160,  4687,  4834,  4860,  5050,  5454,  5488,
  5555,  5665,  5907

Hga1      [    8]   455,   707,   960,  1580,  4175,  4591,
  5169,  5899

HgiA1     [    9]   413,  1721,  2798,  2988,  3828,  4300,
  4798,  5959,  6044

Hha1      [   31]   394,   396,   445,   447,   714,   971,
  2679,  2687,  2751,  2788,  3054,  3084,  3086,
  3314,  3809,  3811,  3862,  4083,  4186,  4216,
  4357,  4390,  4660,  4727,  4827,  5001,  5110,
  5503,  5596,  5933,  6265

HinP1     [   31]   392,   394,   443,   445,   712,   969,
  2677,  2685,  2749,  2786,  3052,  3082,  3084,
  3312,  3807,  3809,  3860,  4081,  4184,  4214,
  4355,  4388,  4658,  4725,  4825,  4999,  5108,
  5501,  5594,  5931,  6263

Hinc2     [    1]  5914

Hind2     [    1]  5914

Hind3     [    1]  2498

Hinf1     [   14]   298,   517,   857,   868,  1553,  1814,
  3170,  3304,  3356,  3881,  4380,  4455,  4851,
  5368

Hpa2      [   30]   161,   237,   473,   601,   643,   789,
  2590,  2667,  2689,  2717,  2848,  2938,  3005,
  3186,  3578,  3653,  3888,  4016,  4058,  4126,
  4160,  4687,  4834,  4860,  5050,  5454,  5488,
  5555,  5665,  5907

Hph1      [   11]  1214,  1240,  1817,  2863,  4102,  4111,
  5216,  5443,  5859,  6065,  6100

Kpn1      [    2]   480,  3895

Mae1      [   15]    30,   293,   689,   727,   739,  1452,
  1606,  1893,  1963,  2483,  3442,  3709,  4975,
  5228,  5563
```

Mae2	[11] 808, 1139, 1180, 1987, 2801, 2988, 4233, 5183, 5599, 5972, 6292
Mae3	[20] 38, 1052, 1080, 1145, 1289, 1478, 1706, 2805, 3111, 3450, 4134, 4229, 4836, 4899, 5015, 5298, 5629, 5687, 5840, 6028
Mbo1	[30] 93, 1102, 1234, 1419, 1657, 2010, 2152, 2521, 2526, 2545, 2856, 2934, 3015, 3024, 3102, 3505, 4019, 5046, 5121, 5132, 5140, 5218, 5230, 5335, 5676, 5694, 5740, 5998, 6015, 6051
Mbo2	[17] 444, 1145, 1356, 1575, 1617, 1908, 1911, 3046, 3256, 3336, 4351, 5142, 5213, 5968, 6046, 6155, 6351
Mnl1	[54] 291, 444, 508, 534, 560, 639, 841, 939, 1227, 1330, 1363, 1369, 1372, 1378, 1408, 1411, 1426, 1433, 1449, 1559, 1620, 1909, 1921, 2412, 2418, 2443, 2449, 2455, 2458, 2470, 2508, 2535, 2599, 2735, 3092, 3286, 3707, 3859, 3878, 3923, 3948, 3974, 4054, 4087, 4117, 4379, 4587, 4662, 4911, 5311, 5392, 5540, 5746, 6339
Mse1	[22] 35, 1065, 1177, 1207, 1231, 1801, 1843, 1956, 1971, 2124, 2139, 3447, 4261, 5186, 5238, 5243, 5257, 5310, 5545, 5584, 5949, 6321
Msp1	[30] 161, 237, 473, 601, 643, 789, 2590, 2667, 2689, 2717, 2848, 2938, 3005, 3186, 3578, 3653, 3888, 4016, 4058, 4126, 4160, 4687, 4834, 4860, 5050, 5454, 5488, 5555, 5665, 5907
Mst1	[2] 2787, 5595
Mst2	[2] 847, 1076
Mva1	[22] 315, 623, 801, 814, 1227, 1252, 1275, 1295, 1325, 1526, 1536, 1558, 1630, 2196, 2251, 2268, 3072, 3731, 4038, 4508, 4629, 4642
Nae1	[1] 3187
Nar1	[2] 2685, 3860
Nci1	[20] 161, 237, 473, 474, 602, 644, 789, 2689, 2849, 3578, 3653, 3888, 3889, 4017, 4059, 4126, 4161, 4860, 5556, 5907
Nco1	[2] 2389, 3117

```
Nde1        [    1]   4303

Nde2        [   30]      93,  1102,  1234,  1419,  1657,  2010,
          2152,  2521,  2526,  2545,  2856,  2934,  3015,
          3024,  3102,  3505,  4019,  5046,  5121,  5132,
          5140,  5218,  5230,  5335,  5676,  5694,  5740,
          5998,  6015,  6051

Nhe1        [    3]      29,  1605,  3441

Nla3        [   26]      61,  1263,  1596,  1649,  1835,  1856,
          2030,  2230,  2302,  2393,  2559,  2904,  3090,
          3121,  3147,  3473,  4119,  4224,  4484,  5204,
          5695,  5705,  5783,  5819,  6212,  6317

Nla4        [   28]     153,   246,   262,   320,   478,   627,
          758,   827,   959,  1202,  1279,  2154,  2200,
          2272,  2686,  2721,  3678,  3736,  3861,  3893,
          4042,  4512,  4551,  5323,  5417,  5458,  5669,
          6259

Nsi1        [    2]   2232,  2304

Nsp(7524)1[    8]   1596,  1835,  1856,  2230,  2302,  3090,
          4119,  4484

Nsp(7524)2[   19]     323,   413,   426,   597,  1583,  1721,
          2631,  2724,  2798,  2988,  3050,  3739,  3828,
          3841,  4012,  4300,  4798,  5959,  6044

NspB2       [   12]     119,   190,  1751,  2158,  2791,  3532,
          3607,  3989,  4192,  4822,  5067,  6008

NspH1       [    8]   1596,  1835,  1856,  2230,  2302,  3090,
          4119,  4484

PaeR7I      [    1]   1998

Pal1        [   35]     171,   202,   247,   658,   792,   828,
          840,  1045,  1169,  1190,  1255,  1534,  1650,
          1866,  1961,  2423,  2429,  2438,  2481,  2593,
          2767,  3158,  3185,  3543,  3588,  3618,  3663,
          4495,  4506,  4524,  4958,  5416,  5496,  5763,
          6350

Ple1        [    7]     865,  1547,  3350,  3889,  4374,  4859,
          5362

PpuM1       [    3]     328,  1277,  3744

Pss1        [    4]     331,  1280,  3747,  6352

Pst1        [    6]     987,  1163,  1888,  2511,  2738,  5618

Pvu1        [    1]   5743
```

Pvu2 [6] 119, 190, 1751, 2791, 3532, 3607

Rsa1 [10] 347, 478, 725, 1342, 1519, 1597,
2991, 3893, 4288, 5853

Rsr2 [1] 3201

Sac1 [2] 413, 3828

Sau1 [2] 847, 1076

Sau3A1 [30] 93, 1102, 1234, 1419, 1657, 2010,
2152, 2521, 2526, 2545, 2856, 2934, 3015,
3024, 3102, 3505, 4019, 5046, 5121, 5132,
5140, 5218, 5230, 5335, 5676, 5694, 5740,
5998, 6015, 6051

Sau96I [29] 169, 200, 245, 260, 273, 328,
626, 756, 826, 839, 1043, 1254, 1277,
1532, 1649, 3201, 3541, 3586, 3616, 3661,
3676, 3689, 3744, 4041, 5415, 5494, 5511,
5733, 6349

Sca1 [1] 5853

ScrF1 [42] 161, 237, 315, 473, 474, 602,
623, 644, 789, 801, 814, 1227, 1252, 1275,
1295, 1325, 1526, 1536, 1558, 1630, 2196,
2251, 2268, 2689, 2849, 3072, 3578, 3653,
3731, 3888, 3889, 4017, 4038, 4059, 4126,
4161, 4508, 4629, 4642, 4860, 5556, 5907

Sdu1 [19] 323, 413, 426, 597, 1583, 1721,
2631, 2724, 2798, 2988, 3050, 3739, 3828,
3841, 4012, 4300, 4798, 5959, 6044

Sec1 [38] 159, 235, 314, 324, 472, 536,
621, 622, 760, 799, 800, 812, 813, 1225,
1294, 1303, 1323, 1324, 1525, 1557, 1962,
2194, 2266, 2389, 2424, 2433, 2482, 2848,
3117, 3576, 3651, 3730, 3740, 3887, 3950,
4036, 4037, 4640

SfaN1 [23] 258, 520, 997, 1657, 2107, 2239,
2311, 2643, 2898, 2984, 3048, 3114, 3323,
3674, 3934, 4146, 4281, 4317, 4357, 4577,
5629, 5820, 6069

Sfi1 [1] 2435

Sma1 [2] 474, 3889

Spe1 [1] 726

Sph1 [4] 1835, 2230, 2302, 3090

Ssp1	[1]	6177
Sst1	[2]	413, 3828
Stu1	[2]	1961, 2481
Sty1	[9]	324, 536, 1303, 1962, 2389, 2482, 3117, 3740, 3950
Taq1	[15]	860, 1096, 1407, 1418, 1660, 1999, 2514, 2798, 2954, 2978, 3014, 3176, 3367, 4580, 6024
Tha1	[19]	392, 394, 445, 969, 971, 1193, 2751, 3052, 3084, 3807, 3809, 4081, 4083, 4186, 4527, 5108, 5438, 5931, 6263
Tth111I	[6]	465, 877, 1275, 2803, 3880, 4227
Xba1	[2]	1892, 3708
Xho1	[1]	1998
Xho2	[11]	2010, 2152, 2521, 2856, 3102, 5121, 5132, 5218, 5230, 5998, 6015
Xma1	[2]	472, 3887
Xma3	[2]	790, 2591
Xmn1	[1]	5972
Xor2	[1]	5743

Table 4

Enzymes which do not cut LXSNRII.L2:

Acc3	Bgl2	Cla1	Hpa1	Nru1	SnaB1
Apa1	Bsm1	Dra3	Mlu1	PflM1	Spl1
Asu2	BspM2	Eco47III	Mro1	Sac2	Sst2
Ban3	BstB1	Esp1	Not1	Sal1	

Table 5

From 1 to 6365. Numbered from position 1.

```
        LXSNRII.L2 -------1000+-------2000+-------3000+-------4000+-------5000+-------6000+----
Mo-MuSV 5' long ter ------->
- [Split]                                    >
1 to 683 of RIIL2                    ________>
neomycin phosphotra                                  =========>
Mo-MuLV 3' long ter                                              ------->
          signal         1                 1
          Aat1      -----------+----------1-----1-----+----------+----------+----------+----
          Aat2      ---------1-+----------+----------+----------+----------+----------+--1-
          Acc1      -----------+----------+----------+----------+--1-------+----------+----
          Acc2      ----21-----2--1-------+--------1--+2--------2-+21---1-----+1---1-----1+--1-
          Acy1      ---------1-+----------+-------1---+---------1-+----------+---------1+--1-
          Afl1      ---3---11--+---1------+----------+--1----21--+1---------+-----1--1--+----
          Afl2      1----------+1---------1----------+----1-----+----------+----------+----
          Afl3      -----------+------1---+----------+----------+-----1----+----------+----
          Aha1      -11--2-2-1-+----------+--------11-+-------11--2112-------1-+-------1---1+----
          Aha2      ---------1-+----------+-------1---+---------1-+----------+---------1+--1-
          Aha3      -----------+----------+----------+----------+----------+--2-------1+----
          Alu1      211-1--12--+-----2--1--2-----2---1-+--1-112--1-+12--1--12--+1-----111--+----
          Alw1      -----------+-1--1--1---+12----2---2+-1---------1-----------+122----1---2----
          AlwN1     --1--------+----------+----------+------11---+---------1+----------+----
          Aoc1      ----------1+1---------+----------+----------+----------+----------+----
          Aoc2      ---111-1---+------1-1--+--------111-11-------12-1---1-----1-+----------11----
          Aos1      -----------+----------+--------1-+----------+----------+------1----+----
          ApaL1     -----------+--------1--+----------+----------+---1-----1-+-----------1----
          Apy1      ---1---1-2-+--23--31---+--3--------+1-------1--+1----1-2---+----------+----
          Aqu1      --1--1-----+----------1----------+---------21+----------+----------+----
          Ase1      -----------+---------1-+----------+----------+----------+------1----+----
          Asp700    -----------+----------+----------+----------+----------+----------1----
          Asp718    -----1-----+----------+----------+----------1+----------+----------+----
          AspA1     -----------+-1--------+----------+----------+----------+----------+----
```

Asu1	--33---112-+1-11--11---+-----------+--1---241--+1----------+----12--1--+---1
Ava1	--1--1-----+-----------1-----------+---------21+-----------+-----------+----
Ava2	---3---11--+---1-------+-----------+--1----21--+1----------+-----1--1--+----
Ava3	-----------+-----------+--11-------+-----------+-----------+-----------+----
Avr2	-----------+-----------1-----1-----+-----------+-----------+-----------+----
Bal1	-------1---+-1---------+--------1--+-----------+-----------+-----------+----
BamH1	-----------+-----------+-1---------+-----------+-----------+-----------+----
Ban1	---1-1-----+--1--------+-------11--+--------111+-----------+---1-------+----
Ban2	----11-1---+------1----+-----------+1--------2-1-----------+-----------+----
Bbe1	-----------+-----------+-------1---+---------1-+-----------+-----------+----
Bbv1	-----------2--------1--+-----1-1121+---1-------+111-2----12+1---1-1--1-+----
Bcl1	-----------+-----------+------1----+-----------+-----------+-----------+----
Bgl1	-----------+-----------+----1------+-----------+-----------+------1----+----
Bsp1286I	---111-1---+------1-1--+-------111-11-------12-1---1-----1-+----------11----
BspH1	-----------+-----------+-----------+-----------+-----------+-1---------+-1-1
BspM1	-----------+----1------+-----11----1-----------+-----------+-----------+----
BssH2	----11-----+-----------+-----------+1--------1-+-----------+-----------+----
BstE2	-----------+-1---------+-----------+-----------+-----------+-----------+----
BstN1	---1---1-2-+--23--31---+--3--------+1-------1--+1----1-2---+-----------+----
BstU1	----21-----2--1--------+--------1--+2-------2--+21---1-----+1---1-----1+--1-
BstX1	-----------+-----------+1----------+-----------+-----------+-----------+----
BstY1	-----------+-----------1-1----1--1-+1----------+-----------+112--------2----
Bsu36I	----------1+1----------+-----------+-----------+-----------+-----------+----
Ccr1	-----------+-----------1-----------+-----------+-----------+-----------+----
Cfo1	----22--1--1-----------+-------211-+3--1-----3-+11111--111-11----11---1+--1-
Cfr1	-------1-1-+-11--------+------1-1--+-2---------+-----------+--------1--+----
Cfr10I	-----------+-----------+----------1-1----------+-----------+----1------+----
Cfr13I	--33---112-+1-11--11---+-----------+--1---241--+1----------+----12--1--+---1
Cvn1	----------1+1----------+-----------+-----------+-----------+-----------+----
Dde1	1111--1---1+1---1---1--+-----1-----+---1-1111-11---1----1--+-1-1-----1-+--1-
Dpn1	-1---------+-11-1--1---1-1----3---212----1-----1-----------+2221---21--21---
Dra1	-----------+-----------+-----------+-----------+-----------+--2-------1+----
Dra2	---1-------+---1-------+-----------+--------1--+-----------+-----------+---1
Eae1	-------1-1-+-11--------+------1-1--+-2---------+-----------+--------1--+----
Eag1	---------1-+-----------+------1----+-----------+-----------+-----------+----
Eco47I	---3---11--+---1-------+-----------+--1----21--+1----------+-----1--1--+----
Eco52I	---------1-+-----------+------1----+-----------+-----------+-----------+----

Eco81I	----------1+1----------+-----------+-----------+-----------+-----------+----
EcoN1	----------1+-----1-----+-----------+-----------+-----------+-----------+----
EcoO109I	---1-------+---1-------+-----------+--------1--+-----------+-----------+---1
EcoR1	-----------+-----1-----+-----------+-----------+-----------+-----------+----
EcoR1*	-----------11----1---21+11111------+-----------+-----------+--1---1--1-+----
EcoR2	---1---1-2-+--23--31---+--3--------+1-------1--+1----1-2---+-----------+----
EcoR5	-11--------+-----------+-----------+------11---+-----------+-----------+----
EcoT22I	-----------+-----------+--11-------+-----------+-----------+-----------+----
Fdi2	-----------+-----------+---------1-+-----------+-----------+------1----+----
Fnu4H1	---------1-3--1-----1--+----1112223+-112-------+112-31-1-12+1---1-1-2-1+1---
FnuD2	----21-----2--1--------+--------1--+2--------2-+21---1-----+1---1-----1+--1-
Fok1	-----1-----+--1-1--1---+---1--1----11---------1+-1---------+---1-1---1-+----
Fsp1	-----------+-----------+---------1-+-----------+-----------+------1----+----
Hae2	-----------+-----------+--------1--+---------1-+---1----1--+-----------+----
Hae3	--3----1-3-+112---11--11----221-1--+-2----22---+-----3-----1----11--1--+---1
Hap2	-11--1-2-1-+-----------+------112111-1----11--1112-----1-2-+1---1111--1+----
Hga1	-----1--1--1-----1-----+-----------+-----------+-1----1----+-1--------1+----
HgiA1	----1------+--------1--+---------1-1---------1-+---1-----1-+----------11---
Hha1	----22--1--1-----------+-------211-+3--1-----3-+11111--111-11----11---1+--1-
HinP1	----22--1--1-----------+-------211-+3--1-----3-+11111--111-11----11---1+--1-
Hinc2	-----------+-----------+-----------+-----------+-----------+----------1+----
Hind2	-----------+-----------+-----------+-----------+-----------+----------1+----
Hind3	-----------+-----------+----1------+-----------+-----------+-----------+----
Hinf1	---1--1---2+------1--1-+-----------+-1-2------1+----11---1-+---1-------+----
Hpa2	-11--1-2-1-+-----------+------112111-1----11--1112-----1-2-+1---1111--1+----
Hph1	-----------+--2------1-+----------1+-----------+2----------+--1-1----1-+2---
Kpn1	-----1-----+-----------+-----------+----------1+-----------+-----------+----
Mae1	1--1----3--+-----1-1--11-----1-----+----1---1--+-----------1--1---1----+----
Mae2	---------1-+-11--------1---------1-1-----------+--1--------+-1----1----1--1-
Mae3	1----------+21-1-1--1--+---------1-+-1---1-----+-11------111---1---2-1-1----
Mbo1	-1---------+-11-1--1---1-1----3--1121----1-----1-----------+2221---21--21---
Mbo2	-----1-----+-1--1-11--2+-----------+1-11-------+---1-------+-11--------2-1-1
Mnl1	---1-131--11--117211--2+----262-1--+1--1----113121--1-11--1+---111--1--+---1
Mse1	1----------+1-3------2-2-2---------+------1----+--1--------+-131--2---1+---1
Msp1	-11--1-2-1-+-----------+------112111-1----11--1112-----1-2-+1---1111--1+----
Mst1	-----------+-----------+---------1-+-----------+-----------+------1----+----
Mst2	----------1+1----------+-----------+-----------+-----------+-----------+----

```
Mva1         ---1---1-2-+--23--31---+--3--------+1-------1--+1----1-2---+-----------+----
Nae1         -----------+-----------+-----------+-1---------+-----------+-----------+----
Nar1         -----------+-----------+-------1---+---------1-+-----------+-----------+----
Nci1         -11--2-2-1-+-----------+--------11-+------11--2112-------1-+-------1---1+----
Nco1         -----------+-----------+----1------+-1---------+-----------+-----------+----
Nde1         -----------+-----------+-----------+-----------+---1-------+-----------+----
Nde2         -1---------+-11-1--1---1-1----3--1121----1-----1-----------+2221---21--21---
Nhe1         1----------+-------1---+-----------+----1------+-----------+-----------+----
Nla3         1----------+---1--11-11+1-111-1---1+12---1-----+-11--1-----+-1-----211-+-1-1
Nla4         -112-1-1-2-1--11-------+-111---11--+--------1111+1----11----+---12--1---+--1-
Nsi1         -----------+-----------+--11-------+-----------+-----------+-----------+----
Nsp(7524)1   -----------+------1--11+--11-------+1----------+-1---1-----+-----------+----
Nsp(7524)2   ---111-1---+------1-1--+--------111-11-------12-1---1-----1-+----------11----
NspB2        -11--------+--------1--+-1-------1-+------2----1-1-------1-+1----------1----
NspH1        -----------+------1--11+--11-------+1----------+-1---1-----+-----------+----
PaeR7I       -----------+-----------1-----------+-----------+-----------+-----------+----
Pal1         --3----1-3-+112---11--11----221-1--+-2----22---+------3-----1----11--1--+---1
Ple1         ----------1+------1----+-----------+---1-------1+----1----1-+---1--------+----
PpuM1        ---1-------+---1-------+-----------+--------1--+-----------+-----------+----
Pss1         ---1-------+---1-------+-----------+--------1--+-----------+-----------+---1
Pst1         ----------1-1---------1+-----1--1--+-----------+-----------+-------1----+----
Pvu1         -----------+-----------+-----------+-----------+-----------+--------1--+----
Pvu2         -11--------+--------1--+----------1-+------2---+-----------+-----------+----
Rsa1         ----11--1--+---1--11---+-----------1----------1+---1-------+----------1-+----
Rsr2         -----------+-----------+-----------+--1--------+-----------+-----------+----
Sac1         ----1------+-----------+-----------+---------1-+-----------+-----------+----
Sau1         ----------1+1----------+-----------+-----------+-----------+-----------+----
Sau3A1       -1---------+-11-1--1---1-1----3--1121----1-----1-----------+2221---21--21---
Sau96I       --33---112-+1-11--11---+-----------+--1--241--+1-----------+----12--1--+---1
Sca1         -----------+-----------+-----------+-----------+-----------+----------1-+----
ScrF1        -111-2-3-3-+--23--31---+--3-----11-+1-----111-2122---1-2-1-+-------1---1+----
Sdu1         ---111-1---+------1-1--+--------111-11-------12-1---1-----1-+----------11----
Sec1         -112-112-5-+--14--2----1--2-31---1-+-1----112-112------1---+-----------+----
SfaN1        ---1--1---1--------1---+-111---1--1111-1---1--1+-112--1-----+-------1-1-+1---
Sfi1         -----------+-----------+----1------+-----------+-----------+-----------+----
Sma1         ----1------+-----------+-----------+----------1+-----------+-----------+----
Spe1         ---------1--+-----------+-----------+-----------+-----------+-----------+----
```

```
Sph1    ---------+-------1-+--11-----+1--------+---------+---------+----
Ssp1    ---------+---------+---------+---------+---------+---------+-1--
Sst1    ----1----+---------+---------+-------1-+---------+---------+----
Stu1    ---------+---------1----1----+---------+---------+---------+----
Sty1    --1--1---+--1------1---11----+-1----1--1---------+---------+----
Taq1    --------1+-1--2-1--1----1--1-3-1--1----+-----1---+---------1----
Tha1    ----21---2--1------+------1--+2------2-+21---1---+1---1---1+--1-
Tth111I ----1---1+--1------+-------1-+--------1+-1-------+---------+----
Xba1    ---------+--------1+---------+------1--+---------+---------+----
Xho1    ---------+---------1---------+---------+---------+---------+----
Xho2    ---------+---------1-1--1--1-+1--------+---------+112------2----
Xma1    ----1----+---------+---------+--------1+---------+---------+----
Xma3    -------1-+---------+----1----+---------+---------+---------+----
Xmn1    ---------+---------+---------+---------+---------+---------1----
Xor2    ---------+---------+---------+---------+---------+------1--+----
```

Table 5

from 1 to 6365. Numbered from position 1.

```
    LXSNRIIL2  -------1000+-------2000+-------3000+-------4000+-------5000+-------6000+----
-MuSV 5' long ter ------->
   (Split)                      >
1 to 683 of RIIL2
neomycin phosphotra             ________>
 -MuLV 3' long ter                         =========>
      signal                                          ------->
                     1          1
      Aat1   -------------+-----------1-----1-----+---------------+--------------+------------+----
      Aat2   ---------1-+------------+------------+------------+--------------+---------------+--1-
      Acc1   -----------+------------+------------+------------+--1---------+-----------------+----
      Acc2   ----21-----2--1--------+--------1--+2--------2-+21---1------+1---1------1+--1-
      Acy1   ---------1-+-----------+-------1---+----------1-+-------------+-----------1+--1-
      Afl1   ---1---11--+---1--------+-------------+--1----21--+1-----------+------1--1--+----
      Afl2   1-----------+1----------1-------------+-----1-----+-------------+------------+----
      Afl3   -----------+------1----+-------------+-------------+-----1-----+-------------+----
      Aha1   -11--2-2-1-+-------------+---------11-+------11--2112--------1-+-------------+----
      Aha2   ---------1-+-------------+--------1---+----------1-+-----------+-------1---1+----
      Aha3   -----------+-------------+-------------+-------------+-------------+-----------1+--1-
      Alu1   211-1--12--+-----2--1--2-----2---1-+--1-112--1-+12--1--12--+2--------1+----
      Alw1   -----------+-1--1--1---+12----2---2+-1---------1-----------+1-----111--+----
      AlwN1  --1--------+-------------+-------------+------11---+-------------+122----1---2----
      Aoc1   ----------1+1----------+-------------+-------------+----------1+-------------+----
      Aoc2   ---111-1---+------1-1--+--------111-11--------11-1---1-----1-+-------------+----
      Aos1   -----------+-------------+---------1-+-------------+-------------+----------11----
      ApaL1  -----------+-------1--+-------------+-------------+-------------+------1-----+----
      Apy1   ---1---1-2-+--21--31---+--3---------+1-------1--+1----1-2---+------------1----
      Aqu1   --1--1-----+----------1-------------+----------21+-------------+-------------+----
      Aso1   -----------+---------1-+-------------+-------------+-------------+-----1----+----
      Asp700 -----------+-------------+-------------+-------------+-------------+-----------1----
```

Table 5 (Cont'd)

from 1 to 6365. Numbered from position 1.

```
           LXSNRIIL2 -------1000+-------2000+-------3000+-------4000+-------5000+-------6000+----
   -MuSV 5' long ter -------->
             (Split)                   >
   1 to 603 of RIIL2                ________>
 neomycin phosphotra                            =========>
   -MuLV 3' long ter                                     ------->
              signal       1              1
              Asp718 -----1-----+----------+----------+----------1+----------+----------+----
              AspA1  ----------+-1--------+----------+----------+----------+----------+----
              Asu1   --33---112-+1-11--11--+----------+--1---241--+1---------+----12--1--+---1
              Ava1   --1--1-----+----------1----------+--------21+----------+----------+----
              Ava2   ---3---11--+---1------+----------+--1----21--+1---------+----1--2--+----
              Ava3   ----------+----------+--11-------+----------+----------+----------+----
              Avr2   ----------+----------1-----1----+----------+----------+----------+----
              Bal1   -------1---+-1--------+--------1--+----------+----------+----------+----
              BamH1  ----------+----------+-1---------+----------+----------+----------+----
              Ban1   ---1-1-----+--1-------+-------11--+---------111+----------+---1------+----
              Ban2   ----11-1---+------1---+----------+1--------2-1----------+----------+----
              Bbe1   ----------+----------+-------1--+--------1-+----------+----------+----
              Bbv1   ----------2-------1--+------1-1121+---1--------+111-2-----12+1---1-1--1-+----
              Bcl1   ----------+----------+-----1----+----------+----------+----------+----
              Bgl1   ----------+----------+----1-----+----------+----------+---1------+----
            Bsp1286I ---111-1---+-----1-1--+-------111-11---------12-1---1-----1-+----------11---
              BspH1  ----------+----------+----------+----------+-1---------+-1-1
              BspM1  ----------+------1---+------11----1----------+----------+----------+----
              BsoM2  ----11-----+----------+----------+1--------1-+----------+----------+----
```

Table 5 (Cont'd)

```
from 1 to 6365.  Numbered from position 1.

      LXSNRIIL2  -------1000+-------2000+-------3000+-------4000+-------5000+-------6000+----
  -MuSV 5' long ter -------->
  [Split]                             >
 1 to 603 of RIIL2                ________>
neomycin phosphotra                          ==========>
  -MuLV 3' long ter                                     -------->
       signal          1          1
       BstE2     ------------+-1---------+-------------+-------------+-------------+-------------+----
       BstN1     ---1---1-2-+--23--31---+--3---------+1--------1--+1----1-2---+-------------+----
       BstU1     ----21-----2--1---------+---------1--+2--------2-+21---1-----+1---1------1+--1-
       BstX1     ------------+------------+1-----------+-------------+-------------+-------------+----
       BstY1     ------------+------------1-1----1--1-+1-----------+-------------+-------------+----
       Bsu36I    -----------1+1-----------+-------------+-------------+112---------2----
       Ccr1      ------------+------------1-------------+-------------+-------------+-------------+----
       Cfo1      ----22--1--1-------------+--------211-+3--1-----3-+11111--111-11----11---1+--1-
       Cfr1      -------1-1-+-11---------+-------1-1--+-2---------+-------------+-------------+----
       Cfr10I    ------------+-------------+-----------1-1---------+-------------+---------1--+----
       Cfr13I    --33---112-+1-11--11---+-------------+--1---241--+1-----------+----1-------+----

       Cva1      -----------1+1-----------+-------------+-------------+-------------+----12--1--+---1
       Dds1      1111--1---1+1---1---1--+------1-----+---1-1111-11---1----1--+-1-1------1-+--1-
       Dpn1      -1----------+-11-1--1---1-1----3---212----1-----1-----------+-------------+----
       Dra1      ------------+-------------+-------------+-------------+2221---21--21---
       Dra2      ---1-------+---1---------+-------------+-------------+--2-------1+----
       Eae1      -------1-1-+-11---------+-------1-1--+-2---------1--+-------------+-------------+----
       Eag1      ---------1-+-------------+-------1----+-------------+-------------+---------1--+---1
       Eco47I    ---3---11--+---1---------+-------------+-------------+-------------+----
       Eco52I    ---------1-+-------------+-------1----+--1----21--+1-----------+-----1--1--+----
       Eco81I    -----------1+1-----------+-------------+-------------+-------------+----
       EcoN1     -----------1+------1-----+-------------+-------------+-------------+----
       EcoO109I  ---1-------+---1---------+-------------+-------------+-------------+----
       EcoR1     ------------+------1------+-------------+--------1--+-------------+-------------+---1
       EcoR1*    -----------11----1---21+11111---------+-------------+-------------+--1---1--1-+----
       EcoR2     ---1---1-2-+--23--31---+--3---------+1--------1--+1----1-2---+-------------+----
       EcoR5     -11---------+-------------+-------------+------11---+-------------+-------------+----
```

Table 5 (Cont'd)

from 1 to 6365. Numbered from position 1.

```
         LXSNRIIL2  -------1000+--------2000+--------3000+--------4000+--------5000+--------6000+----
  -MuSV 5' long ter -------->
          (Split)
    1 to 683 of RIIL2                  >
  neomycin phosphotra              --------->
     -MuLV 3' long ter                          =========>
            signal        1          1                      ------->
           EcoT22I  -----------+-----------+--11-------+-----------+-----------+-----------+----
              Fdi2  -----------+-----------+---------1-+-----------+-----------+-----------+----
            Fnu4H1  ---------1-3--1-----1--+----1112223+-112-------+112-31-1-12+1---1----+----
             FnuD2  ----21-----2--1--------+---------1--+2--------2-+21---1------+1---1-1-2-1+1---
              Fok1  -----1-----+--1-1--1---+---1--1----11---------1+-1---------+---1-----1+--1-
              Fsp1  -----------+-----------+---------1-+-----------+-----------+---1-1---1-+----
              Hae2  -----------+-----------+---------1-+-----------+-----------+-------1----+----
              Hae3  --3----1-3-+112---11--11----221-1--+-3----22---+-----3-----1--+-----------+----
              Hap2  -11--1-2-1-+-----------+-------112111-1----11--1112-----1-2-+1---11--1--+---1
              Hga1  -----1--1--1------1----+-----------+-----------+-1----1----+-1---1111--1+----
             HgiA1  ----1------+-----1-----1--+---------1-1---------1-+---1-----1-+----------1+----
              Hha1  ----22--1--1-----------+--------211-+3--1------3-+11111--111-11-----11---1+--1-
             HinP1  ----22--1--1-----------+--------211-+3--1------3-+11111--111-11-----11---1+--1-
             Hinc2  -----------+-----------+-----------+-----------+-----------+-----------1+----
             Hind2  -----------+-----------+-----------+-----------+-----------+-----------1+----
             Hind3  -----------+-----------+-----1-----+-----------+-----------+-----------+----
             Hinf1  ---1--1---2+------1--1-+-----------+-1-2------1+----11---1--+--1--------+----
              Hpa2  -11--1-2-1-+-----------+-------112111-1----11--1112-----1-2-+1---1111--1+----
              Hph1  -----------+--2-------1-+----------1+-----------+2----------+--1-1----1-+2---
              Kpn1  -----1-----+-----------+-----------+-----------1+-----------+-----------+----
              Mae1  1--1----3--+-----1-1--11------1----+-----1---1--+-----------1--1---1----+----
              Mae2  ---------1-+-11--------1----------1-1---------+--1---------+-1-----1----1--1-
              Mae3  1----------+21-1-1--1--+---------1-+-1---1------+-11--------111----1----2-1-1-
              Mbo1  -1---------+-11-1--1---1-1-----3--1121---1------1-----------+2221---21---21--
              Mbo2  ----1------+--1--1-11--2+-----------+1-11---------+--1-------+-11-----------2-1-1
              Mnl1  ---1-131--11--117211---2+-----262-1--+1--1----111121---1-11--1+---111--1--+---1
              Mse1  1----------+1-1------2-2-2---------+-----1-----+--1---------+-131--2---1+---1
              Msp1  -11--1-2-1-+-----------+-------112111-1----11--1112-----1-2-+1---1111--1+----
```

Table 5 (Cont'd)

from 1 to 6365. Numbered from position 1.

```
      LXSHRIIL2  -------1000+--------2000+--------3000+--------4000+--------5000+--------6000+----
 -MuSV 5' long ter -------->
  [Split]                             >
1 to 683 of RIIL2                 ---------->
Neomycin phosphotra                        ---------->
  -MuLV 3' long ter                                  -------->
      signal         1                  1

     Nat1      -----------+-------+-----+---------1-+-----------+------------+------1----+----
     Nat2      ----------1+1----------+-----------+------------+------------+-----------+----
     Nva1      ---1---1-2-+--23--31---+--3--------+1-------1--+1----1-2---+-----------+----
     Nae1      -----------+-----------+-----------+-1---------+------------+-----------+----
     Nar1      -----------+-----------+-------1---+---------1-+------------+-----------+----
     Nci1      -11--2-2-1-+-----------+--------11-+------11--2112-------1-+------1---1+----
     Nco1      -----------+-----------+----1------+-1---------+------------+-----------+----
     Nde1      -----------+-----------+-----------+-----------+---1--------+-----------+----
     Nde2      -1---------+-11-1--1---1-1----3--1121----1-----1-----------+2221---21--21---
     Nhe1      1----------+-------1---+-----------+----1------+------------+-----------+----
     Nla3      1----------+---1--11-11+1-111-1---1+12---1-----+-11--1-----+-1-----211-+-1-1
     Nla4      -112-1-1-2-1--11-------+-111---11--+-------1111+1----11----+---12--1---+--1-
     Nsi1      -----------+-----------+--11-------+-----------+------------+-----------+----
   Nsp(7524)1------------+------1--11+--11-------+1----------+-1---1------+-----------+----
   Nsp(7524)2---111-1---+-------1-1--+--------111-11--------12-1---1-----1-+----------11----
     NspB2     -11--------+--------1--+-1-------1-+-------2----1-1-------1-+1----------1----
     NspH1     -----------+-------1--11+--11------+1----------+-1---1------+-----------+----
     PaeR71    -----------+----------1------------+-----------+------------+-----------+----
     Pal1      --3----1-3-+112---11--11----221-1--+-2----22---+-----3-----1----11--1--+---1
     Ple1      ----------1+-------1----+-----------+---1------1+----1----1-+---1--------+----
     PpuM1     ---1-------+---1--------+-----------+---------1--+-----------+-----------+----
     Pss1      ---1-------+---1--------+-----------+---------1--+-----------+-----------+----
     Pst1      -----------1-1---------1+-----1--1--+-----------+------------+--------+----1
     Pvu1      -----------+-----------+-----------+-----------+------------+------1----+----
     Pvu2      -11--------+--------1--+----------1-+-------2----+------------+-----------+----
```

Table 5 (Cont'd)

from 1 to 6365. Numbered from position 1.

```
      LXSNRIIL2  -------1000+--------2000+--------3000+--------4000+--------5000+--------6000+----
  -MuSV 5' long ter --------> 
  (Split)                              >
 1 to 683 of RIIL2                ______>
neomycin phosphotra                              ==========>
  -MuLV 3' long ter                                        -------->
         signal          1                  1
         Rsal     ----11--1--+---1--11---+-----------1----------1+---1--------+---------1-+----
         Rsr2     -----------+-------------+----------+--1--------+-----------+---------+----
         Sac1     ----1------+-------------+----------+---------1-+-----------+---------+----
         Sau1     ----------1+1------------+----------+-----------+-----------+---------+----
         Sau3A1   -1---------+-11-1--1---1-1----3--1121----1-----1-----------+2221---21--21---
         Sau96I   --33---112-+1-11--11---+-----------+--1---241--+1----------+----12--1--+---1
         Sca1     -----------+-------------+----------+-----------+-----------+---------1-+----
         ScrF1    -111-2-3-3-+--23--31---+--3-----11-+1-----111-2122---1-2-1-+------1---1+----
         Sdu1     ---111-1---+-------1-1--+--------111-11--------12-1---1-----1-+-----------11----
         Sec1     -112-112-5-+--14--2----1--2-21---1-+-1----112-112------1---+-----------+----
         SfaN1    ---1--1----1-------1---+-111---1--1111-1---1--1+-112--1----+--------1-1-+1---
         Sfi1     -----------+-------------+----1------+-----------+-----------+---------+----
         Sma1     -----1-----+-------------+-----------+---------1+-----------+---------+----
         Spe1     --------1--+-------------+-----------+-----------+-----------+---------+----
         Sph1     -----------+---------1-+--11--------+1----------+-----------+---------+----
         Ssp1     -----------+-------------+-----------+-----------+-----------+---------+----
         Sst1     ---1-------+-------------+-----------+---------1-+-----------+---------+-1--
         Stu1     -----------+------------1-----1------+-----------+-----------+---------+----
         Sty1     ---1--1----+---1--------1----11------+-1-------1--1----------+---------+----
         Taq1     ----------1+-1--2--1---1------1---1-3-1--1--------+--------1----------+----
         Tha1     ---21------2--1----------+-----------1--+2---------2-+21---1------+---------1----
         Tth111I  -----1----1+---1---------+-----------1-+----------1+--1----------+---------+----
         Xba1     -----------+-----------1+-----------+---------1--+-----------+---------+----
         Xho1     -----------+------------1------------+-----------+-----------+---------+----
         Xho2     -----------+------------1-1----1--1--+1----------+-----------+112---------2----
         Xma1     -----1-----+-------------+-----------+---------1+-----------+---------+----
         Xma3     ---------1-+-------------+----1------+-----------+-----------+---------+----
         Xmn1     -----------+-------------+-----------+-----------+-----------+---------1----
         Xor2     -----------+-------------+-----------+-----------+-----------+------1--+----
```

Table 6

from 1 to 6365. Numbered from position 1.

```
                                          >Nhe1      >Afl2
mouse_DNA_end/MO-MuSV_DNA_start_(Split)
              10         20         30         40         50         60         70
TTTGAAAGAC CCCACCCGTA GGTGGCAAGC TAGCTTAAGT AACGCCACTT TGCAAGGCAT GGAAAAATAC
AAACTTTCTG GGGTGGGCAT CCACCGTTCG ATCGAATTCA TTGCGGTGAA ACGTTCCGTA CCTTTTTATG

                                                  >Pvu2
                                                  >NspB2                 >EcoR5
          80         90        100        110        120        130        140
ATAACTGAGA ATAGGAAAGT TCAGATCAAG GTCAGGAACA AAGAAACAGC TGAATACCAA ACAGGATATC
TATTGACTCT TATCCTTTCA AGTCTAGTTC CAGTCCTTGT TTCTTTGTCG ACTTATGGTT TGTCCTATAG

                                                  >Pvu2
                                                  >NspB2
         150        160        170        180        190        200        210
TGTGGTAAGC GGTTCCTGCC CCGGCTCAGG GCCAAGAACA GATGAGACAG CTGAGTGATG GGCCAAACAG
ACACCATTCG CCAAGGACGG GGCCGAGTCC CGGTTCTTGT CTACTCTGTC GACTCACTAC CCGGTTTGTC

                                  >Ava1
>EcoR5                  >AlwN1    >Aqu1
           220        230        240        250        260        270        280
GATATCTGTG GTAAGCAGTT CCTGCCCCGG CTCGGGGCCA AGAACAGATG GTCCCCAGAT GCGGTCCAGC
CTATAGACAC CATTCGTCAA GGACGGGGCC GAGCCCCGGT TCTTGTCTAC CAGGGGTCTA CGCCAGGTCG

                                                 >PpuM1
                                                 >Dra2
                                               >Eco0109I
                                      >Ban1    >Sty1    >Psa1            >Rsa1
         290        300        310        320        330        340        350
CCTCAGCAGT TTCTAGTGAA TCATCAGATG TTTCCAGGGT GCCCCAAGGA CCTGAAAATG ACCCTGTACC
GGAGTCGTCA AAGATCACTT AGTAGTCTAC AAAGGTCCCA CGGGGTTCCT GGACTTTTAC TGGGACATGG
```

Table 6 (Cont'd)

```
                                                                            >Ban2
                                                                            >Sac1
                                                                            >Sst1
                                                        >BssH2              >HgiA1
        360        370        380        390        400        410        420
TTATTTGAAC TAACCAATCA GTTCGCTTCT CGCTTCTGTT CGCGCGCTTC CGCTCTCCGA GCTCAATAAA
AATAAACTTG ATTGGTTAGT CAAGCGAAGA GCGAAGACAA GCGCGCGAAG GCGAGAGGCT CGAGTTATTT

                                                               >Asp718
                                                               >Ban1
                                                              >Sma1
                                                            >Ava1
                                                           >Aqu1   >Rsa1
  >Ban2              >BssH2      >Hga1     >Tth111I        >Xma1   >Kpn1
        430        440        450        460        470        480        490
AGAGCCCACA ACCCCTCACT CGGCGCGCCA GTCTTCCGAT AGACTGCGTC GCCCGGGTAC CCGTATTCCC
TCTCGGGTGT TGGGGAGTGA GCCGCGCGGT CAGAAGGCTA TCTGACGCAG CGGGCCCATG GGCATAAGGG

                                                >Sty1
        500        510        520        530        540        550        560
AATAAAGCCT CTTGCTGTTT GCATCCGAAT CGTGGTCTCG CTGTTCCTTG GGAGGGTCTC CTCTGAGTGA
TTATTTCGGA GAACGACAAA CGTAGGCTTA GCACCAGAGC GACAAGGAAC CCTCCCAGAG GAGACTCACT

                                      >Ban2
        570        580        590        600        610        620        630
TTGACTACCC ACGACGGGGG TCTTTCATTT GGGGGCTCGT CCGGGATTTG GAGACCCCTG CCCAGGGACC
AACTGATGGG TGCTGCCCCC AGAAAGTAAA CCCCCGAGCA GGCCCTAAAC CTCTGGGGAC GGGTCCCTGG

                             >Bal1
```

Table 6 (Cont'd)

```
                                  >CfrI
                                  .
                                  >EaeI
                                  .
                           >extended_packaging_signal
      640         650          660        670        680        690        700
   .   .        .    .        *v vv*    .    .      .    .     .    .      .   .
ACCGACCCAC CACCGGGAGG TAAGCTGGCC AGCAACTTAT CTGTGTCTGT CCGATTGTCT AGTGTCTATG
TGGCTGGGTG GTGGCCCTCC ATTCGACCGG TCGTTGAATA GACACAGACA GGCTAACAGA TCACAGATAC
```

```
                        >SpeI
                        .
  >HgaI                 >RsaI
  .                     .
      710         720          730        740        750        760        770
 .  v   .       .    .        *v   .    .    .      .    .     .    .      .   .
TTTGATGTTA TGCGCCTGCG TCTGTACTAG TTAGCTAACT AGCTCTGTAT CTGGCGGACC CGTGGTGGAA
AAACTACAAT ACGCGGACGC AGACATGATC AATCGATTGA TCGAGACATA GACCGCCTGG GCACCACCTT
```

```
                   >Eco52I
                   .
                   >CfrI
                   .                           >AatII
                   >XmaIII                     .
                   .                      >NaeII
                   >EagI                  .
                   .                      >AhaII
                   >EaeI                  .
                   .                      >AcyI
      780          790        800         810        820        830        840
   .    .        .    .     .    .      .  v  .  v    .    .     .    .      .   .
CTGACGAGTT CTGAACACCC GGCCGCAACC CTGGGAGACG TCCCAGGGAC TTTGGGGGCC GTTTTTGTGG
GACTGCTCAA GACTTGTGGG CCGGCGTTGG GACCCTCTGC AGGGTCCCTG AAACCCCCGG CAAAAACACC
```

```
     >EcoNI
     .
  >Bsu36I
  .
  >AocI
  .
  >SauI
  .
 >Eco81I
 .
```

Table 6 (Cont'd)

```
  >Cvn1|
   .   |
  >Mst2|                 >Ple1        >Tth111I
   .   |                   .              .
   |850           860      |   870        |880          890           900          910
 . v  .          .   .      .     .      . v  .        .    .        .    .        .    .
CCCGACCTGA GGAAGGGAGT CGATGTGGAA TCCGACCCCG TCAGGATATG TGGTTCTGGT AGGAGACGAG
GGGCTGGACT CCTTCCCTCA GCTACACCTT AGGCTGGGGC AGTCCTATAC ACCAAGACCA TCCTCTGCTC

                                                         >Hga1
                                                           .
       920        930         940         950         960         970         980
  .    .      .    .      .     .      .    .      .    .      .    .       .    .
AACCTAAAAC AGTTCCCGCC TCCGTCTGAA TTTTTGCTTT CGGTTTGGAA CCGAAGCCGC GCGTCTTGTC
TTGGATTTTG TCAAGGGCGG AGGCAGACTT AAAAACGAAA GCCAAACCTT GGCTTCGGCG CGCAGAACAG

  >Pst1
    .
    |990       1000        1010        1020        1030        1040        1050
  . v  .      .    .      .    .      .    .      .    .      .    .      .    .
TGCTGCAGCA TCGTTCTGTG TTGTCTCTGT CTGACTGTGT TTCTGTATTT GTCTGAAAAT TAGGGCCAGA
ACGACGTCGT AGCAAGACAC AACAGAGACA GACTGACACA AAGACATAAA CAGACTTTTA ATCCCGGTCT

                            >Aoc1
                              .
                            >Sau1
                              .
                            >Cvn1
                              .
                            >Mst2
                              .
                           >Bsu36I
                              .
              >Afl2        >Eco81I
                .             .
      1060      |  1070       |1080        1090        1100        1110        1120
  .    .        v.    .      .v    .      .    .      .    .      .    .      .    .
CTGTTACCAC TCCCTTAAGT TTGACCTTAG GTCACTGGAA AGATGTCGAG CGGATCGGTC ACAACCAGTC
GACAATGGTG AGGGAATTCA AACTGGAATC CAGTGACCTT TCTACAGCTC GCCTAGCCAG TGTTGGTCAG

                                                >Cfr1
                                                  .
                           >AspA1               >Eae1                   >Eae1
                              .                   .                       .
```

Table 6 (Cont'd)

```
                   >Mae2     >BstE2                   >Pst1   |>Bal1      >Mae2    >Cfr1
    1130           1140       | 1150          1160       |    1170        1180        1190
GGTAGATGTC AAGAAGAGAC GTTGGGTTAC CTTCTGCTCT GCAGAATGGC CAACCTTTAA CGTCGGATGG
CCATCTACAG TTCTTCTCTG CAACCCAATG GAAGACGAGA CGTCTTACCG GTTGGAAATT GCAGCCTACC

       >Ban1            >Hph1                               >Hph1
    1200           1210       | 1220          1230          1240          1250          1260
CCGCGAGACG GCACCTTTAA CCGAGACCTC ATCACCCAGG TTAAGATCAA GGTCTTTTCA CCTGGCCCGC
GGCGCTCTGC CGTGGAAATT GGCTCTGGAG TAGTGGGTCC AATTCTAGTT CCAGAAAAGT GGACCGGGCG

                  >Pss1
             >Dra2|
           >EcoO109I
             >PpuM1
          >Tth111I
    1270       | 1280         1290          1300    >Sty1  1310          1320          1330
ATGGACACCC AGACCAGGTC CCCTACATCG TGACCTGGGA AGCCTTGGCT TTTGACCCCC CTCCCTGGGT
TACCTGTGGG TCTGGTCCAG GGGATGTAGC ACTGGACCCT TCGGAACCGA AAACTGGGGG GAGGGACCCA

         >Rsa1
    1340 |         1350          1360          1370          1380          1390          1400
CAAGCCCTTT GTACACCCTA AGCCTCCGCC TGCTGTTCCT CCATCCGCCC CGTCTCTCCC CCTTGAACCT
GTTCGGGAAA CATGTGGGAT TCGGAGGCGG ACGACAAGGA GGTAGGCGGG GCAGAGAGGG GGAACTTGGA

                                                             >EcoN1        >EcoR1
    1410           1420          1430          1440          1450          1460          1470
GCTCGTTCGA CCCCCGCTCG ATCCTCCCTT TATCCAGCCC TCACTCCTTC TCTAGGCCGG AATTCGTTAG
CGAGCAAGCT GGGGGCGAGC TAGGAGGGAA ATAGGTCGGG AGTGAGGAAG AGATCCGGCC TTAAGCAATC
```

Table 6 (Cont'd)

```
                                     >NspH1                       >Rsa1
       1480          1490        1500      1510         1520          1530         1540
CTTGGTAAGT GACCAGCTAC AGTCGGAAAC CATCAGCAAG CAGGTATGTA CTCTCCAGGG TGGGCCTGGC
GAACCATTCA CTGGTCGATG TCAGCCTTTG GTAGTCGTTC GTCCATACAT GAGAGGTCCC ACCCGGACCG
```

```
                                                                  >Rsa1
                                                                 >NspH1
                                               >Ban2        >Nsp(7524)1
   >Ple1                                 >Hga1          >Afl3           >Nhe1
      1550         1560         1570         1580         1590      1600         1610
TTCCCCAGTC AAGACTCCAG GGATTTGAGG GACGCTGTGG GCTCTTCTCT TACATGTACC TTTTGCTAGC
AAGGGGTCAG TTCTGAGGTC CCTAAACTCC CTGCGACACC CGAGAAGAGA ATGTACATGG AAAACGATCG
```

```
      1620         1630         1640         1650         1660         1670         1680
CTCAACCCTG ACTATCTTCC AGGTCATTGT TCCAACATGG CCCTGTGGAT CGACAGGATG CAACTCCTGT
GAGTTGGGAC TGATAGAAGG TCCAGTAACA AGGTTGTACC GGGACACCTA GCTGTCCTAC GTTGAGGACA
```

```
                                              >HgiA1
                                      >ApaL1
      1690         1700         1710         1720         1730         1740         1750
CTTGCATTGC ACTAAGTCTT GCACTTGTCA CAAACAGTGC ACCTACTTCA AGTTCTACAA AGAAAACACA
GAACGTAACG TGATTCAGAA CGTGAACAGT GTTTGTCACG TGGATGAAGT TCAAGATGTT TCTTTTGTGT
```

```
>Pvu2
>NspB2                                                      >Ase1          >Hph1
      1760         1770         1780         1790         1800         1810         1820
GCTGCAACTG GAGCATTTAC TGCTGGATTT ACAGATGATT TTGAATGGAA TTAATAATTA CAAGAATCCC
CGACGTTGAC CTCGTAAATG ACGACCTAAA TGTCTACTAA AACTTACCTT AATTATTAAT GTTCTTAGGG
```

```
              >Sph1
          >Nsp(7524)1                 >NspH1
              >NspH1               >Nsp(7524)1                               >Pst1
      1830         1840         1850      1860         1870         1880         1890
AAACTCACCC GCATGCTCAC ATTTAAGTTT TACATGCCCA AGAAGGCCAC AGAACTGAAA CATCTGCAGT
TTTGAGTGGG CGTACGAGTG TAAATTCAAA ATGTACGGGT TCTTCCGGTG TCTTGACTTT GTAGACGTCA
```

Table 6 (Cont'd)

```
>Xba1                                                                   >Afl2

   1900       1910       1920       1930       1940       1950       1960
GTCTAGAAGA AGAACTCAAA CCTCTGGACG AAGTGCTAAA TTTAGCTCAA AGCAAAAACT TTCACTTAAG
CAGATCTTCT TCTTGAGTTT GGAGACCTCC TTCACGATTT AAATCGAGTT TCGTTTTTGA AAGTGAATTC

                                        >Ava1
>Avr2                                   >Aqu1
>Sty1                                   >Ccr1
>Stu1                                   >PaeR7I       >Xho2
>Aat1                          >Hae2    >Xho1         >BstY1

       1970       1980       1990       2000       2010       2020       2030
GCCTAGGGAC TTAATCAGCA ATATCAACGT AATAGTTCTC GAGCTAAAGG GATCTGAAAC AACATTCATG
CGGATCCCTG AATTAGTCGT TATAGTTGCA TTATCAAGAG CTCGATTTCC CTAGACTTTG TTGTAAGTAC

                              >BstX1

      2040       2050       2060       2070       2080       2090       2100
TGTGAAATATG CTGATGAGAC AGCCACCATT GTGGAATTTC TCAACAGATG GATTACCTTT TGTCAAAGCA
ACACTTATAC GACTACTCTG TCGGTGGTAA CACCTTAAAG ACTTGTCTAC CTAATGGAAA ACAGTTTCGT

                                                  >BamH1
                                                  >BstY1
                                                 >Xho2     >Nsp B2
                                              >simian_virus_40_early_promoter
                                         >Mo-MuSV_DNA_end/simian_virus_40_DNA_start

      2110       2120       2130       2140       2150       2160       2170
TCATCTCAAC ACTAACTTGA TAATTAAGTG CTTCCCACTT AAAACATATC AGGATCCGCT GTGGAATGTG
AGTAGAGTTG TGATTGAACT ATTAATTCAC GAAGGGTGAA TTTTGTATAG TCCTAGGCGA CACCTTACAC

                                                                 >EcoT22I
                                                                 >Nsi1
                                                                 >Ava3
```

Table 6 (Cont'd)

```
                                                                  >Nsp(7524)I
                                                                  >NspHI
                                                                  >SphI
      2180       2190       2200       2210       2220       2230       2240
TGTCAGTTAG GGTGTGGAAA GTCCCCAGGC TCCCCAGCAG GCAGAAGTAT GCAAAGCATG CATCTCAATT
ACAGTCAATC CCACACCTTT CAGGGGTCCG AGGGGTCGTC CGTCTTCATA CGTTTCGTAC GTAGAGTTAA
```

```
                                                                  >NsiI
                                                                  >Ava3
                                                                  >EcoT22I
                                                                  >Nsp(7524)I
                                                                  >NspHI
                                                                  >SphI
      2250       2260       2270       2280       2290       2300       2310
AGTCAGCAAC CAGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT ATGCAAAGCA TGCATCTCAA
TCAGTCGTTG GTCCACACCT TTCAGGGGTC CGAGGGGTCG TCCGTCTTCA TACGTTTCGT ACGTAGAGTT
```

```
      2320       2330       2340       2350       2360       2370       2380
TTAGTCAGCA ACCATAGTCC CGCCCCTAAC TCCGCCCATC CCGCCCCTAA CTCCGCCCAG TTCCGCCCAT
AATCAGTCGT TGGTATCAGG GCGGGGATTG AGGCGGGTAG GGCGGGGATT GAGGCGGGTC AAGGCGGGTA
```

```
   >NcoI                                              >SfiI
   >StyI                                              >BglI
      2390       2400       2410       2420       2430       2440       2450
TCTCCGCCCC ATGGCTGACT AATTTTTTTT ATTTATGCAG AGGCCGAGGC CGCCTCGGCC TCTGAGCTAT
AGAGGCGGGG TACCGACTGA TTAAAAAAAA TAAATACGTC TCCGGCTCCG GCGGAGCCGG AGACTCGATA
```

```
                    >StyI
                    >Avr2
                    >StuI                         >BspMI
```

Table 6 (Cont'd)

```
                                    >Aat1                        >Hind3           >Pst1
       2460         2470        2480         2490        2500        2510        2520
TCCAGAAGTA GTGAGGAGGC TTTTTTGGAG GCCTAGGCTT TTGCAAAAAG CTTGGGCTGC AGGTCGAGGC
AGGTCTTCAT CACTCCTCCG AAAAAACCTC CGGATCCGAA AACGTTTTTC GAACCCGACG TCCAGCTCCG

   >Bcl1
>Xho2
>BstY1                                                          >BspM1
Lmian_virus_DNA_end/Tn5_DNA_start
        2530        2540        2550        2560        2570        2580
GGATCTGATC AAGAGACAGG ATGAGGATCG TTTCGC ATG ATT GAA CAA GAT GGA TTG CAC GCA GGT TCT
CCTAGACTAG TTCTCTGTCC TACTCCTAGC AAAGCG TAC TAA CTT GTT CTA CCT AAC GTG CGT CCA AGA
                                        Met Ile Glu Gln Asp Gly Leu His Ala Gly Ser>

>Eco52I
>Eag1
>Xma1
>Cfr1
>Xma3
2590        2600        2610        2620        2630        2640        2650
CCG GCC GCT TGG GTG GAG AGG CTA TTC GGC TAT GAC TGG GCA CAA CAG ACA ATC GGC TGC TCT
GGC CGG CGA ACC CAC CTC TCC GAT AAG CCG ATA CTG ACC CGT GTT GTC TGT TAG CCG ACG AGA
Pro Ala Ala Trp Val Glu Arg Leu Phe Gly Tyr Asp Trp Ala Gln Gln Thr Ile Gly Cys Ser>

                          >Hae2
                          >Bbe1
                        >Nar1
                        >Acy1
                        >Aha2
```

Table 6 (Cont'd)

```
                                       >BanI
   2660         2670        2680               2690          2700          2710
GAT GCC GCC GTG TTC CGG CTG TCA GCG CAG GGG CGC CCG GTT CTT TTT GTC AAG ACC GAC CTG
CTA CGG CGG CAC AAG GCC GAC AGT CGC GTC CCC GCG GGC CAA GAA AAA CAG TTC TGG CTG GAC
Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe Val Lys Thr Asp Leu>
```

```
                                                                      >BalI
                                                                   >CfrI
 >BanI                     >PstI                                   >EaeI
  2720        2730          2740         2750         2760            2770
TCC GGT GCC CTG AAT GAA CTG CAG GAC GAG GCA GCG CGG CTA TCC TGG CTG GCC ACG ACG GGC
AGG CCA CGG GAC TTA CTT GAC GTC CTG CTC CGT CGC GCC GAT AGG ACC GAC CGG TGC TGC CCG
Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly>
```

```
        >FspI
        >AosI               >Tth111I
       >Fdi2   >Pvu2    >HgiA1
       >MstI   >NspB2      >Mae2
2780       2790      2800         2810         2820          2830         2840
GTT CCT TGC GCA GCT GTG CTC GAC GTT GTC ACT GAA GCG GGA AGG GAC TGG CTG CTA TTG GGC
CAA GGA ACG CGT CGA CAC GAG CTG CAA CAG TGA CTT CGC CCT TCC CTG ACC GAC GAT AAC CCG
Val Pro Cys Ala Ala Val Leu Asp Val Val Thr Glu Ala Gly Arg Asp Trp Leu Leu Leu Gly>
```

```
             >BstYI
             >XhoI   >HphI
   2850          2860         2870         2880         2890         2900
GAA GTG CCG GGG CAG GAT CTC CTG TCA TCT CAC CTT GCT CCT GCC GAG AAA GTA TCC ATC ATG
CTT CAC GGC CCC GTC CTA GAG GAC AGT AGA GTG GAA CGA GGA CGG CTC TTT CAT AGG TAG TAC
Glu Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys Val Ser Ile Met>

                                                           >BspMI
```

Table 6 (Cont'd)

```
  2910      2920      2930      2940      2950      2960
GCT GAT GCA ATG CGG CGG CTG CAT ACG CTT GAT CCG GCT ACC TGC CCA TTC GAC CAC CAA GCG
CGA CTA CGT TAC GCC GCC GAC GTA TGC GAA CTA GGC CGA TGG ACG GGT AAG CTG GTG GTT CGC
Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro Ala Thr Cys Pro Phe Asp His Gln Ala>
```

```
                          >RsaI
                      >HgiAI
                      >Hae2                        >Cfr10I
2970      2980      2990      3000      3010      3020      3030
AAA CAT CGC ATC GAG CGA GCA CGT ACT CGG ATG GAA GCC GGT CTT GTC GAT CAG GAT GAT CTG
TTT GTA GCG TAG CTC GCT CGT GCA TGA GCC TAC CTT CGG CCA GAA CAG CTA GTC CTA CTA GAC
Lys His Arg Ile Glu Arg Ala Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu>
```

```
                                                                  >SphI
                                                              >Nsp(7524)I
                      >Ban2                                 >BssH2  >NspHI
          3040      3050      3060      3070      3080      3090
GAC GAA GAG CAT CAG GGG CTC GCG CCA GCC GAA CTG TTC GCC AGG CTC AAG GCG CGC ATG CCC
CTG CTT CTC GTA GTC CCC GAG CGC GGT CGG CTT GAC AAG CGG TCC GAG TTC CGC GCG TAC GGG
Asp Glu Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala Arg Met Pro>
```

```
        >Xho2             >NcoI                                           >CfrI
        >BstY1            >StyI                                           >EaeI
      3100      3110      3120      3130      3140      3150
GAC GGC GAG GAT CTC GTC GTG ACC CAT GGC GAT GCC TGC TTG CCG AAT ATC ATG GTG GAA AAT
CTG CCG CTC CTA GAG CAG CAC TGG GTA CCG CTA CGG ACG AAC GGC TTA TAG TAC CAC CTT TTA
Asp Gly Glu Asp Leu Val Val Thr His Gly Asp Ala Cys Leu Pro Asn Ile Met Val Glu Asn>
```

Table 6 (Cont'd)

```
                                             >Cfr10I
                                          >CfrI
                                        >EaeI  >NaeI              >Rsr2
 3160        3170        3180                3190          3200      3210
GGC CGC TTT TCT GGA TTC ATC GAC TGT GGC CGG CTG GGT GTG GCG GAC CGC TAT CAG GAC ATA
CCG GCG AAA AGA CCT AAG TAG CTG ACA CCG GCC GAC CCA CAC CGC CTG GCG ATA GTC CTG TAT
Gly Arg Phe Ser Gly Phe Ile Asp Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile>

3220         3230        3240         3250          3260        3270         3280
GCG TTG GCT ACC CGT GAT ATT GCT GAA GAG CTT GGC GGC GAA TGG GCT GAC CGC TTC CTC GTG
CGC AAC CGA TGG GCA CTA TAA CGA CTT CTC GAA CCG CCG CTT ACC CGA CTG GCG AAG GAG CAC
Ala Leu Ala Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe Leu Val>

       3290         3300         3310         3320         3330         3340
CTT TAC GGT ATC GCC GCT CCC GAT TCG CAG CGC ATC GCC TTC TAT CGC CTT CTT GAC GAG TTC
GAA ATG CCA TAG CGG CGA GGG CTA AGC GTC GCG TAG CGG AAG ATA GCG GAA GAA CTG CTC AAG
Leu Tyr Gly Ile Ala Ala Pro Asp Ser Gln Arg Ile Ala Phe Tyr Arg Leu Leu Asp Glu Phe>

   >PleI
              >Tn5_DNA_end/_Mo-MuLV_DNA_start
   3350       3360       3370       3380       3390       3400       3410       3420
TTC TGA GCGGGACTC TGGGGTTCGA TAAAATAAAA CATTTTATTT AGTCTCCAGA AAAAGGGGGG AATGAAAGAC
AAG ACT CGCCCTGAG ACCCCAAGCT ATTTTATTTT GTAAAATAAA TCAGAGGTCT TTTTCCCCCC TTACTTTCTG
Phe End>

                          >AflII
                   >NheI
       3430       3440       3450       3460       3470       3480       3490
CCCACCTGTA GGTTTGGCAA GCTAGCTTAA GTAACGCCAT TTTGCAAGGC ATGGAAAAAT ACATAACTGA
GGGTGGACAT CCAAACCGTT CGATCGAATT CATTGCGGTA AAACGTTCCG TACCTTTTTA TGTATTGACT

                                          >NspB2
                                          >Pvu2                         >EcoR5
       3500       3510       3520       3530       3540       3550       3560
GAATAGAGAA GTTCAGATCA AGGTCAGGAA CAGATGGAAC AGCTGAATAT GGGCCAAACA GGATATCTGT
CTTATCTCTT CAAGTCTAGT TCCAGTCCTT GTCTACCTTG TCGACTTATA CCCGGTTTGT CCTATAGACA
```

Table 6 (Cont'd)

```
                                                       >Nsp B2
            >AlwN1                                      >Pvu2                 >EcoR5
     3570          3580        3590        3600         3610        3620        3630
GGTAAGCAGT TCCTGCCCCG GCTCAGGGCC AAGAACAGAT GGAACAGCTG AATATGGGCC AAACAGGATA
CCATTCGTCA AGGACGGGGC CGAGTCCCGG TTCTTGTCTA CCTTGTCGAC TTATACCCGG TTTGTCCTAT
```

```
             >AlwN1
     3640        3650        3660        3670        3680        3690        3700
TCTGTGGTAA GCAGTTCCTG CCCCGGCTCA GGGCCAAGAA CAGATGGTCC CCAGATGCGG TCCAGCCCTC
AGACACCATT CGTCAAGGAC GGGGCCGAGT CCCGGTTCTT GTCTACCAGG GGTCTACGCC AGGTCGGGAG
```

```
                                                  >Ppu1
                                               >Dra2
                                             >Eco0109I
                                               >PpuM1
     >Xba1                         >Ban1   >Sty1
      3710        3720        3730        3740        3750        3760        3770
AGCAGTTTCT AGAGAACCAT CAGATGTTTC CAGGGTGCCC CAAGGACCTG AAATGACCCT GTGCCTTATT
TCGTCAAAGA TCTCTTGGTA GTCTACAAAG GTCCCACGGG GTTCCTGGAC TTTACTGGGA CACGGAATAA
```

```
                                                             >Sac1
                                                             >HglA1
                                                  >Aqu1      >Sst1
                                  >BssH2          >Ava1      >Ban2
     3780        3790        3800        3810        3820        3830        3840
TGAACTAACC AATCAGTTCG CTTCTCGCTT CTGTTCGCGC GCTTCTGCTC CCCGAGCTCA ATAAAAGAGC
ACTTGATTGG TTAGTCAAGC GAAGAGCGAA GACAAGCGCG CGAAGACGAG GGGCTCGAGT TATTTTCTCG
```

```
                                                  >Ban1
                   >Hae2                          >Asp718
                   >Hha1                        >Ple1
```

Table 6 (Cont'd)

```
                                    >Har1
                                  >Ban1
                  >Aqu1           >Acy1                                       >Sma1
 >Ban2            >Ava1           >Aha2                                     >Xma1
                                                                            >Ava1   >Kpn1
                                                      >Tth111I              >Aqu1   >Rsa1
             3850           3860            3870            3880            3890            3900            3910
 CCACAACCCC TCACTCGGGG CGCCAGTCCT CCGATTGACT GAGTCGCCCG GGTACCCGTG TATCCAATAA
 GGTGTTGGGG AGTGAGCCCC GCGGTCAGGA GGCTAACTGA CTCAGCGGGC CCATGGGCAC ATAGGTTATT
```

```
                                                   >Sty1
       3920       3930        3940        3950        3960        3970        3980
 ACCCTCTTGC AGTTGCATCC GACTTGTGGT CTCGCTGTTC CTTGGGAGGG TCTCCTCTGA GTGATTGACT
 TGGGAGAACG TCAACGTAGG CTGAACACCA GAGCGACAAG GAACCCTCCC AGAGGAGACT CACTAACTGA
```

```
     >NspB2
                                   >Ban2
      3990        4000        4010        4020        4030        4040        4050
 ACCCGTCAGC GGGGGTCTTT CATTTGGGGG CTCGTCCGGG ATCGGGAGAC CCCTGCCCAG GGACCACCGA
 TGGGCAGTCG CCCCCAGAAA GTAAACCCCC GAGCAGGCCC TAGCCCTCTG GGGACGGGTC CCTGGTGGCT
```

```
                                                                              >Nsp(7524)1
                                                              >Nph1     >Nph1   >NspH1
   >Mo-MuLV_DNA_end/plasmid_pBR322_DNA_start
      4060        4070        4080        4090        4100        4110        4120
 CCCACCACCG GGAGGTAAGC TGGCTGCCTC GCGCGTTTCG GTGATGACGG TGAAAACCTC TGACACATGC
 GGGTGGTGGC CCTCCATTCG ACCGACGGAG CGCGCAAAGC CACTACTGCC ACTTTTGGAG ACTGTGTACG
```

```
                                                              >Bgl1
      4130        4140        4150        4160        4170        4180        4190
 AGCTCCCGGA GACGGTCACA GCTTGTCTGT AAGCGGATGC CGGGAGCAGA CAAGCCCGTC AGGGCGCGTC
 TCGAGGGCCT CTGCCAGTGT CGAACAGACA TTCGCCTACG GCCCTCGTCT GTTCGGGCAG TCCCGCGCAG
```

```
>NspB2
                                      >Tth111I    >Hae2
                                                                    >Acc1
```

Table 6 (Cont'd)

4200 4210 4220 4230 4240 4250 4260

AGCGGGTGTT GCCGGGTGTC GGGGCGCAGC CATGACCCAG TCACGTAGCG ATAGCGGAGT GTATACTGGC
TCGCCCACAA CGGCCCACAG CCCCGCGTCG GTACTGGGTC AGTGCATCGC TATCGCCTCA CATATGACCG

>HgiA1

>Rsa1 >ApaL1 >Nde1

4270 4280 4290 4300 4310 4320 4330

TTAACTATGC GGCATCAGAG CAGATTGTAC TGAGAGTGCA CCATATGCGG TGTGAAATAC CGCACAGATG
AATTGATACG CCGTAGTCTC GTCTAACATG ACTCTCACGT GGTATACGCC ACACTTTATG GCGTGTCTAC

>Hae2 >Ple1

4340 4350 4360 4370 4380 4390 4400

CGTAAGGAGA AAATACCGCA TCAGGCGCTC TTCCGCTTCC TCGCTCACTG ACTCGCTGCG CTCGGTCGTT
GCATTCCTCT TTTATGGCGT AGTCCGCGAG AAGGCGAAGG AGCGAGTGAC TGAGCGACGC GAGCCAGCAA

4410 4420 4430 4440 4450 4460 4470

CGGCTGCGGC GAGCGGTATC AGCTCACTCA AAGGCGGTAA TACGGTTATC CACAGAATCA GGGGATAACG
GCCGACGCCG CTCGCCATAG TCGAGTGAGT TTCCGCCATT ATGCCAATAG GTGTCTTAGT CCCCTATTGC

>Nsp(7524)1

>NspH1

>Afl3

4480 4490 4500 4510 4520 4530 4540

CAGGAAAGAA CATGTGAGCA AAAGGCCAGC AAAAGGCCAG GAACCGTAAA AAGGCCGCGT TGCTGGCGTT
GTCCTTTCTT GTACACTCGT TTTCCGGTCG TTTTCCGGTC CTTGGCATTT TTCCGGCGCA ACGACCGCAA

>Hga1

4550 4560 4570 4580 4590 4600 4610

Table 6 (Cont'd)

```
TTTCCATAGG CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA GTCAGAGGTG GCGAAACCCG
AAAGGTATCC GAGGCGGGGG GACTGCTCGT AGTGTTTTTA GCTGCGAGTT CAGTCTCCAC CGCTTTGGGC

        4620      4630      4640      4650      4660      4670      4680
ACAGGACTAT AAAGATACCA GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT CCGACCCTGC
TGTCCTGATA TTTCTATGGT CCGCAAAGGG GGACCTTCGA GGGAGCACGC GAGAGGACAA GGCTGGGACG

                                            >Hae2
        4690      4700      4710      4720      4730      4740      4750
CGCTTACCGG ATACCTGTCC GCCTTTCTCC CTTCGGGAAG CGTGGCGCTT TCTCATAGCT CACGCTGTAG
GCGAATGGCC TATGGACAGG CGGAAAGAGG GAAGCCCTTC GCACCGCGAA AGAGTATCGA GTGCGACATC

                                             >HgiA1
                                         >ApaL1
        4760      4770      4780      4790      4800      4810      4820
GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC TGTGTGCACG AACCCCCCGT TCAGCCCGAC
CATAGAGTCA AGCCACATCC AGCAAGCGAG GTTCGACCCG ACACACGTGC TTGGGGGGCA AGTCGGGCTG

>NspB2                             >Ple1
        4830      4840      4850      4860      4870      4880      4890
CGCTGCGCCT TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA CGACTTATCG CCACTGGCAG
GCGACGCGGA ATAGGCCATT GATAGCAGAA CTCAGGTTGG GCCATTCTGT GCTGAATAGC GGTGACCGTC

 >AlwN1
        4900      4910      4920      4930      4940      4950      4960
CAGCCACTGG TAACAGGATT AGCAGAGCGA GGTATGTAGG CGGTGCTACA GAGTTCTTGA AGTGGTGGCC
GTCGGTGACC ATTGTCCTAA TCGTCTCGCT CCATACATCC GCCACGATGT CTCAAGAACT TCACCACCGG

        4970      4980      4990      5000      5010      5020      5030
TAACTACGGC TACACTAGAA GGACAGTATT TGGTATCTGC GCTCTGCTGA AGCCAGTTAC CTTCGGAAAA
ATTGATGCCG ATGTGATCTT CCTGTCATAA ACCATAGACG CGAGACGACT TCGGTCAATG GAAGCCTTTT

                             >NspB2
```

```
      5040      5050      5060      5070      5080      5090      5100
AGAGTTGGTA GCTCTTGATC CGGCAAACAA ACCACCGCTG GTAGCGGTGG TTTTTTTGTT TGCAAGCAGC
TCTCAACCAT CGAGAACTAG GCCGTTTGTT TGGTGGCGAC CATCGCCACC AAAAAAACAA ACGTTCGTCG

                    >Xho2     >BstY1
                    >BstY1    >Xho2                                    >Hga1
      5110      5120      5130      5140      5150      5160      5170
AGATTACGCG CAGAAAAAAA GGATCTCAAG AAGATCCTTT GATCTTTTCT ACGGGGTCTG ACGCTCAGTG
TCTAATGCGC GTCTTTTTTT CCTAGAGTTC TTCTAGGAAA CTAGAAAAGA TGCCCCAGAC TGCGAGTCAC

                                          >BstY1
                                          >Xho2        >BstY1     >Dra1
           >Hae2          >BspH1          >Hph1        >Xho2      >Aha3
      5180      5190      5200      5210      5220      5230      5240
GAACGAAAAC TCACGTTAAG GGATTTTGGT CATGAGATTA TCAAAAAGGA TCTTCACCTA GATCCTTTTA
CTTGCTTTTG AGTGCAATTC CCTAAAACCA GTACTCTAAT AGTTTTTCCT AGAAGTGGAT CTAGGAAAAT

                 >Dra1
                 >Aha3
      5250      5260      5270      5280      5290      5300      5310
AATTAAAAAT GAAGTTTTAA ATCAATCTAA AGTATATATG AGTAAACTTG GTCTGACAGT TACCAATGCT
TTAATTTTTA CTTCAAAATT TAGTTAGATT TCATATATAC TCATTTGAAC CAGACTGTCA ATGGTTACGA

          >Ban1                                       >Ple1
      5320      5330      5340      5350      5360      5370      5380
TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTCG TTCATCCATA GTTGCCTGAC TCCCCGTCGT
ATTAGTCACT CCGTGGATAG AGTCGCTAGA CAGATAAAGC AAGTAGGTAT CAACGGACTG AGGGGCAGCA

                                                                 >Hph1
      5390      5400      5410      5420      5430      5440      5450
GTAGATAACT ACGATACGGG AGGGCTTACC ATCTGGCCCC AGTGCTGCAA TGATACCGCG AGACCCACGC
CATCTATTGA TGCTATGCCC TCCCGAATGG TAGACCGGGG TCACGACGTT ACTATGGCGC TCTGGGTGCG

>Cfr10I                                      >Bgl1
          5460      5470      5480      5490      5500      5510      5520
TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCAGCCG GAAGGGCCGA GCGCAGAAGT GGTCCTGCAA
AGTGGCCGAG GTCTAAATAG TCGTTATTTG GTCGGTCGGC CTTCCCGGCT CGCGTCTTCA CCAGGACGTT
```

Table 6 (Cont'd)

```
                         >Ase1
                           |
       5530      5540      5550      5560      5570      5580      5590
CTTTATCCGC CTCCATCCAG TCTATTAATT GTTGCCGGGA AGCTAGAGTA AGTAGTTCGC CAGTTAATAG
GAAATAGGCG GAGGTAGGTC AGATAATTAA CAACGGCCCT TCGATCTCAT TCATCAAGCG GTCAATTATC

     >Hae2
   >Aos1
   >Fsp1
   >Fdi2
   >Mst1                      >Pst1
   |   5600      5610      5620      5630      5640      5650      5660
TTTGCGCAAC GTTGTTGCCA TTGCTGCAGG CATCGTGGTG TCACGCTCGT CGTTTGGTAT GGCTTCATTC
AAACGCGTTG CAACAACGGT AACGACGTCC GTAGCACCAC AGTGCGAGCA GCAAACCATA CCGAAGTAAG

       5670      5680      5690      5700      5710      5720      5730
AGCTCCGGTT CCCAACGATC AAGGCGAGTT ACATGATCCC CCATGTTGTG CAAAAAAGCG GTTAGCTCCT
TCGAGGCCAA GGGTTGCTAG TTCCGCTCAA TGTACTAGGG GGTACAACAC GTTTTTTCGC CAATCGAGGA

           >Pvu1                >Eae1
           >Xor2                >Cfr1
       5740 |    5750      5760 |    5770      5780      5790      5800
TCGGTCCTCC GATCGTTGTC AGAAGTAAGT TGGCCGCAGT GTTATCACTC ATGGTTATGG CAGCACTGCA
AGCCAGGAGG CTAGCAACAG TCTTCATTCA ACCGGCGTCA CAATAGTGAG TACCAATACC GTCGTGACGT

                                                      >Rsa1
                                                      >Sca1   >Hph1
       5810      5820      5830      5840      5850   |  5860      5870
TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG AGTACTCAAC CAAGTCATTC
ATTAAGAGAA TGACAGTACG GTAGGCATTC TACGAAAAGA CACTGACCAC TCATGAGTTG GTTCAGTAAG
```

Table 6 (Cont'd)

```
                                                          >Hinc2
                                                          >Hind2
                                                >Acy1
                                 >Hga1          >Aha2
      5880       5890       5900       5910       5920       5930       5940
TGAGAATAGT GTATGCGGCG ACCGAGTTGC TCTTGCCCGG CGTCAACACG GGATAATACC GCGCCACATA
ACTCTTATCA CATACGCCGC TGGCTCAACG AGAACGGGCC GCAGTTGTGC CCTATTATGG CGCGGTGTAT

                                  >Asp700
       >Aha3                      >Mae2
       >Dra1      >HgiA1          >Xmn1                          >BstY1
                                                                 >Xho2     >Nsp82
      5950       5960       5970       5980       5990       6000       6010
GCAGAACTTT AAAAGTGCTC ATCATTGGAA AACGTTCTTC GGGGCGAAAA CTCTCAAGGA TCTTACCGCT
CGTCTTGAAA TTTTCACGAG TAGTAACCTT TTGCAAGAAG CCCCGCTTTT GAGAGTTCCT AGAATGGCGA

>Xho2                             >HgiA1
>BstY1                  >ApaL1                         >Hph1
      6020       6030       6040       6050       6060       6070       6080
GTTGAGATCC AGTTCGATGT AACCCACTCG TGCACCCAAC TGATCTTCAG CATCTTTTAC TTTCACCAGC
CAACTCTAGG TCAAGCTACA TTGGGTGAGC ACGTGGGTTG ACTAGAAGTC GTAGAAAATG AAAGTGGTCG

                 >Hph1
      6090       6100       6110       6120       6130       6140       6150
GTTTCTGGGT GAGCAAAAAC AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA CGGAAATGTT
CAAAGACCCA CTCGTTTTTG TCCTTCCGTT TTACGGCGTT TTTTCCCTTA TTCCCGCTGT GCCTTTACAA

                            >Ssp1                               >BspM1
      6160       6170       6180       6190       6200       6210       6220
```

Table 6 (Cont'd)

```
GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT TATTGTCTCA TGAGCGGATA
CTTATGAGTA TGAGAAGGAA AAAGTTATAA TAACTTCGTA AATAGTCCCA ATAACAGAGT ACTCGCCTAT

        6230      6240      6250      6260      6270      6280      6290
CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCGCACAT TTCCCCGAAA AGTGCCACCT
GTATAAACTT ACATAAATCT TTTTATTTGT TTATCCCCAA GGCGCGTGTA AAGGGGCTTT TCACGGTGGA

   >Aat2
>Aha2
>Acy1                                                                   >Pss1
>Hae2                                                             >Eco0109I
                       >BspM1                                     >Dra2
          6300      6310      6320      6330      6340      6350      6360
GACGTCTAAG AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGCG TATCACGAGG CCCTTTCGTC
CTGCAGATTC TTTGGTAATA ATAGTACTGT AATTGGATAT TTTTATCCGC ATAGTGCTCC GGGAAAGCAG

TTCAA
AAGTT
```

ymes which do not cut LXSNRIIL2 :

Acc3	Bgl2	Cla1	Hpa1	Nru1	SnaB1
Apa1	Bsm1	Dra3	Mlu1	PflM1	Spl1
Asu2	BspM2	Eco47III	Mro1	Sac2	Sst2
Ban3	BstB1	Esp1	Not1	Sal1	

To generate the LXSN-RI-IL2 retroviral vector, 10 micrograms of pLXSN-RI-IL2 DNA was transfected into the ecotropic packaging cell line PE501 by standard calcium phosphate precipitation methods (Miller et al., Mol. Cell Biol. 6:2895, 1986). The transfected PE501 cell line was grown in DMEM medium with 10% FCS. The medium was changed after 24 hours and supernatant harvested 24 hours later to infect the amphotropic packaging cell line PA317 as described (Miller et al., Mol. Cell Biol. 6:2895, 1986 and Miller et al., BioTechniques 7:980, 1989). The infected PA317 cells were harvested by trypsinization 24 hours later and replated 1:20 in DMEM containing 10% FCS and the neomycin analogue G418 (400 μg/ml). The cells were grown at 37°C in 7% CO_2 atmosphere. The selection medium was changed every 5 days until colonies appeared. On day 14, twenty colonies were selected, expanded and tested for viral production by standard methods (Xu et al., Virology 171:331-341, 1989). Briefly, supernatants were harvested from confluent culture dishes, passed through a .45 μm filter, diluted with DMEM with 10% FCS and utilized to infect NIH 3T3 cells in the presence of 8 μg/ml polybrene. After 24 hours, the infected NIH 3T3 cells were grown in culture medium that contained the neomycin analogue G418. After 12-14 days, the colonies were stained, counted and the viral titer calculated as described (Xu et al., Virology 171:331-341, 1989).

Colonies with the highest viral titers ($>10^4$ infectious units/ml) were tested for IL-2 expression by Northern blot analyses. Colonies with the highest viral titers and documented IL-2 expression were cryopreserved and will be utilized as stock cultures to produce the LXSN-RI-IL2 retroviral vector trial.

EXAMPLE IV

RETROVIRAL VECTOR CONSTRUCTION AND CYTOKINE EXPRESSION

To increase IL-2 production by transduced cell lines, vectors were used containing different promoters to drive IL-2 expression, and a human IL-2 cDNA was directionally sub-cloned into the insulin secretory signal peptide (17). The IL-2 cDNA was directionally sub-cloned into the parental plasmids of the LXSN (LTR promoter) and LNCX (CMV promoter) vectors (gifts of Dr. A.D. Miller) (18). The newly constructed vectors (Figure 1), designated as LXSN-IL2 and LNCX-IL2, were packaged in the PA317 cell line for production of retroviral supernatant. As a control, the high level expressing, double copy vector DC/TKIL-2 vector (thymidine kinase promoter) (a gift of Dr. E. Gilboa) was used for comparison.

These vectors were used to transduce a number of murine and human, primary and established cell lines. Pools of transduced cells were selected and expanded in DMEM medium, containing 10% fetal bovine serum (FBS) and 400 μg/ml of active G-418, a neomycin analogue. The results of expression studies in the MCR9 and Balb/c 3T3 cell lines are presented in Table 7.

Table 7

Comparison of IL-2 expression by fibroblasts transduced with different IL-2 vectors.

Fibroblast	Vector	ng IL-2 per 10^6 cells per day	Units IL-2 per day
Murine	LNCX (Control)	0.4 ±50%	<1
	LNCX-IL2	33.7 ±11%	67
	LXSN-IL2	6.6 ± 6%	13
	DC/TKIL-2	1.9 ± 5%	4
Human	LXSN (Control)	0.7 ±29%	1
	LNCX-IL2	159.5 ±17%	319
	LXSN-IL2	25.5 ±15%	51
	DC/TKIL-2	3.0 ±10%	6

EXAMPLE V

FIBROBLAST CULTURE AND CONDITIONS FOR RETROVIRAL TRANSDUCTION

The culture conditions for the growth of primary fibroblasts retroviral transduction were optimized. Primary fibroblasts were successfully cultured. The optimal conditions enable the growth of approximately 3-4 x 10^6 primary fibroblasts from a 12 mm^2 skin biopsy in approximately 4-6 weeks. Retroviral infection, G418 selection, and expansion of the genetically modified fibroblasts takes an additional 4-6 weeks.

Exploring the conditions for genetic modification of primary fibroblasts suggests that optimal transduction may be obtained by the following procedure: The fibroblasts are synchronized in G1 phase by serum starvation, followed by stimulation with medium containing 15% fetal bovine serum 15 hours prior to transduction. The cells are then subjected to 2 cycles of retrovirus infection, each cycle lasting approximately 3 hours. The cells are refed with fresh media overnight, and then selection in G418 is initiated the next day. This method is capable of transducing 5-15% of the fibroblasts in a culture, depending on the multiplicity of infection.

This procedure was used to transduce a large number of primary and established fibroblasts. As an example, Table 8 compares the expression levels of IL-2 in fibroblast lines transduced with LXSN-IL2.

Table 8

Expression of IL-2 by fibroblasts transduced with LXSN-IL2.

Fibroblast Line	Species	Origin	ng IL-2 per 10^6 cells	Units IL-2 per day
Balb/c 3T3	Murine	Transformed	6.6 ± 6%	13
MCR9	Human	Embryonic	25.5 ±15%	51
NHDF 313	Human	Skin	25.0 ±10%	50
GT1	Human	Skin	15.0 ± 5%	30

These results indicate that the IL-2 expression levels in established, embryonic, and primary fibroblast cultures are similar. Comparison of these data with Table 7 suggest that IL-2 expression is affected more by factors such as different promoters than by the fibroblast line used. Similarly, changes in culture conditions can have important effects on IL-2 expression. Table 9 shows that transduced GT1 cells, a primary human fibroblast culture expressed 15-fold more IL-2 under 100 μg/ml G418 selection than under 25 μg/ml G418 selection. Several other primary fibroblast lines have also been transduced with our vectors and are currently growing under G418 selection.

Table 9

Effect of G418 concentration on IL-2 expression by GT1 cells transduced with LXSN-IL2.

Selection dose of G418	ng IL-2 secreted per 10^6 cells per day*
25 μg/ml	1.0 ± 10%
50 μg/ml	3.0 ± 6%
100 μg/ml	15.0 ± 5%

*After three weeks of G418 selection.

EXAMPLE VI

COMPARISON OF IL-2 EXPRESSION LEVELS INDUCED PERIPHERAL BLOOD LYMPHOCYTES AND GENETICALLY MODIFIED FIBROBLASTS

In order to compare the production of IL-2 by genetically modified fibroblasts to that achieved by stimulating normal human peripheral blood lymphocytes (nPBL) *in vitro*, nPBL were isolated by Ficol-Paque density centrifugation, and cultured in the presence of allogeneic nPBL (mixed lymphocyte culture, MLC) or 2 μM calcium ionophore (CI) (A23187) free acid) plus 17 nM phorbol 12-myristate 13-acetate (PMA). The results of this experiment, present in Table 10, indicate that the level of IL-2 expression in the PMA/CI stimulated normal T cell population was 2 ng/10^6 cells/24 hours. This is equivalent to IL-2 expression by Balb/c 3T3 fibroblasts transduced with DC/TKIL-2 (Table 7), our least productive vector. The level of IL-2 expression in the MLC was 130 pg/10^6 cells/24 hours. This was lower than the PMA/CI stimulated culture, presumably because PMA/CI induced a nonspecific response

while MLC resulted in specific Th stimulation. When the estimated percentage of antigen-specific Th in the MLC-stimulated population is taken into consideration, the level of IL-2 expression per stimulated T cell becomes equivalent for both methods.

Table 10
Levels of IL-2 secretion by different cells.

Cells	pg IL-2 secreted per 10^6 cells per day
Lymphocytes:	
Control (non-activated)	5 ± 50%
PMA + Calcium Ionophore	2,000 ± 6%
Mixed lymphocyte culture	130 ± 90%
Transduced fibroblasts:	
MCR9-LXSN-IL2	24,000 ± 5%
MCR9-LNCX-IL2	162,000 ± 20%
MCR9-DC/TKIL-2	10,000 ± 6%

EXAMPLE VII

FIBROBLAST MEDIATED CYTOKINE GENE THERAPY IN MURINE TUMOR MODELS

Two experimental protocols were used to study the efficacy of fibroblast-mediated cytokine gene therapy on induction of anti-tumor immunity. The first protocol was designed to test the effects of genetically modified fibroblasts on tumor implantation, while the second protocol was designed to induce a systemic anti-tumor immunity. The results of each experiment are presented with two figures and one table. In the first figure, the rate of tumor growth for each treatment group is presented

as the mean tumor size in the group over time. In the second figure, a Kaplan-Meier curve presents the time of tumor onset for the individual animals in each treatment group. The number of animals, the number and percentage of tumor free animals, and the tumor size distribution patterns for each experiment are presented in a table.

EXAMPLE VII(a)

EFFECT OF FIBROBLAST MEDIATED CYTOKINE GENE THERAPY ON TUMOR IMPLANTATION

Mice were injected subcutaneously with mixtures of 5×10^4 CT26 cells and 2×10^6 fibroblasts genetically modified by different retroviral vectors to express IL-2. In the control arms injected with tumor cells only, or with tumor cells mixed with unmodified fibroblasts, 31 of 33 animals (94%) developed tumors by 4 weeks (Figures 6 and 7, Table 9). In contrast, 22 out of the 34 animals (65%) receiving fibroblast mediated cytokine gene therapy were tumor free at 3 weeks, and 5 animals (18%) remain tumor free after 12 weeks. Those animals that received fibroblast mediated IL-2 therapy and developed tumor were characterized by a delayed onset and rate of tumor growth.

Table 11

Effect of IL-2 modified fibroblasts on tumor establishment and development. 2 X 10^6 fibroblasts mixed with 5 X 10^4 CT26 tumor cells at time of injection.

Fibroblasts mixed with tumor cells	Animal Number: Total	Animal Number: Tumor-free	Animal Number: Tumor-bearing	Percent Tumor-free	Tumor Size (mm^2): 25-100	Tumor Size (mm^2): 101-200	Tumor Size (mm^2): 201-300	Tumor Size (mm^2): >301	Median Tumor Size (mm^2)
After 12 Weeks:*									
Control (no fibroblasts)	11	0	11	0%	1	0	1	9	420 ± 145
Unmodified fibroblasts**	13	2	11	15%	1	0	1	7	388 ± 265
DCTK-IL2 fibroblasts	13	0	13	0%	1	3	5	4	267 ± 168
LNCX-IL2 fibroblasts	13	5	8	39%	5	2	0	1	72 ± 90

* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

** Two mice in this arm developed intraperitoneal tumors which were not measurable.

After 3 weeks the mean tumor size (measured as the product of the longest and widest tumor axes) in the control group of mice was 128 mm^2, compared to 68 and 7 mm^2 in groups of mice injected with tumor cells mixed with fibroblasts transduced with DC/TKIL-2 or LNCX-IL2, respectively. This resulted in a highly significant difference (corrected $x^2 = 18.69$, $p = 0.001$) between the IL-2 treated animals compared to the mice treated with CT26 alone or CT26 mixed with unmodified fibroblasts. After four weeks the equivalent measurements were 373,300 and 72 mm^2 (Table 11). It is notable that LNCX-IL2, the highest expressing vector caused substantially greater inhibition of tumorigenicity than the lower expressing vector DC/TKIL-2. A multivariate non-parametric statistical procedure (19,20), utilized to evaluate differences in tumor growth, demonstrated that after 4 weeks the differences between the growth curves for the four groups presented in Figure 2 were highly significant ($p < 0.001$). Subsequent comparisons between the control arm and animals that received tumor cells mixed with IL-2 transduced fibroblasts revealed a significant difference ($P < 0.05$). The differences between the animals injected with tumor cells alone, and those injected with tumor cells plus unmodified fibroblasts were not significant, while the differences between animals receiving low IL-2 expressing fibroblast, and those receiving high IL-2 expressing fibroblasts was significant ($P = 0.05$).

When mice were injected with 2 x 10^6 modified fibroblasts mixed with 1 x 10^5 live tumor cells the results became more striking (see Figures 8 and 9, and Table 12). All the control animals developed tumors after 4 weeks whereas 33% and 27% of the animals treated with fibroblasts modified with the DCTK-IL2 or LXSN-IL2 vectors (respectively) remain tumor free after 7 weeks (the experiment is ongoing). More dramatically, 75% of the animals treated with fibroblasts modified with the highest

IL-2 producing vector, LNCX-IL2, remain tumor free after 7 weeks. These data clearly demonstrate the importance of an initial high dose of IL-2 to prevent tumor establishment.

Table 12

Effect of IL-2 modified fibroblasts on tumor establishment and development.
2 X 10^6 fibroblasts mixed with 1 X 10^5 CT26 tumor cells at time of injection.

Fibroblasts mixed with tumor cells	Animal Number: Total	Tumor-free	Tumor-bearing	Percent Tumor-free	Tumor Size (mm^2): 25-100	101-200	201-300	>301	Mean Tumor Size (mm^2)
After 6 Weeks:*									
Control (no fibroblasts)**	13	0	13	0%	0	5	2	5	315 ± 197
Unmodified fibroblasts**	20	0	20	0%	0	2	3	14	350 ± 100
DCTK-IL2 fibroblasts	12	4	8	33%	0	1	4	3	185 ± 141
LXSN-IL2 fibroblasts***	15	4	11	27%	0	5	1	2	135 ± 121
LNCX-IL2 fibroblasts	8	6	2	75%	2	0	0	0	8 ± 14

* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

** One mouse in each of these arms developed an intraperitoneal tumor which was not measurable.

*** Three mice in this arm developed intraperitoneal tumors which were not measurable.

As an additional control, mice were injected with CT26 cells genetically modified to express IL-2 (results not shown). Injection of up to 1 x 10^6 IL-2 expressing tumor cells into Balb/c mice failed to produce tumors. Injection of higher numbers however, resulted in some animals developing tumors with delayed onset. These data confirm the results reported in the literature (1). In order to compare the efficacy of IL-2 producing fibroblasts to IL-2 producing tumor cells, we mixed 2 x 10^6 CT26 tumor cells modified with the DCTK-IL2 vector with 1 x 10^5 unmodified tumor cells. Figures 10 and 11, and Table 13 show that DCTK-IL2 modified tumor cells are somewhat effective in preventing tumor development. Four weeks after injection, the mean tumor size for the treatment arm is 303 mm^2, compared to 620 mm^2 for the control arm. After 22 weeks, one animal (10%) remains tumor free, compared to none in the control arms. Data for animals treated under the same conditions with DCTK-IL2 modified fibroblasts in a separate experiment are included for comparison purposes. This comparison suggests that DCTK-IL2 modified tumor cells have an effect on tumor establishment similar to that of DCTK-IL2 modified fibroblasts.

Table 13

Effect of IL-2 modified cells on tumor establishment and development.
2 X 10^6 DCTK-IL2-modified CT26 tumor cells mixed with 1 X 10^5 CT26 cells compared to 2 X 10^6 DCTK-IL2-modified fibroblasts mixed with 1 X 10^5 CT26.

Cells mixed with tumor cells	Animal Number: Total	Tumor-free	Tumor-bearing	Percent Tumor-free	Tumor Size (mm^2): 25-100	101-200	201-300	>301	Mean Tumor Size (mm^2)
After 22 Weeks:*									
Control (no fibroblasts)	5	0	5	0%	0	0	0	5	620 ± 190
Unmodified fibroblasts	5	0	5	0%	0	0	0	5	587 ± 69
DCTK-IL2-modifed CT26 cells	10	1	9	10%	1	0	2	5	303 ± 179
DCTK-IL2-modified fibroblasts	8	2	6	25%	0	1	2	3	214 ± 158

* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

EXAMPLE VII(b)

EFFECT OF FIBROBLAST MEDIATE CYTOKINE GENE THERAPY ON SYSTEMIC ANTI-TUMOR IMMUNITY

Groups of Balb/c mice were immunized with 2.5×10^5 irradiated tumor cells either alone or mixed with 2×10^6 transduced or unmodified fibroblasts, and challenged one week later with 5×10^4 live tumor cells in the opposite flank. These results (Figures 12 and 13, and Table 14) demonstrate that immunization with irradiated tumor cells and transduced fibroblasts protect some animals against a live tumor challenge, but that the protection is only slightly better than that achieved by immunization with irradiated tumor cells alone or irradiated tumor cells mixed with unmodified fibroblasts.

Table 14

Effect of IL-2 modified fibroblasts on induction of sytemic anti-tumor immunity.
Mice immunized with 2 X 10^6 fibroblasts mixed with 2.5 X 10^5 irradiated CT26 tumor cells 7 days prior to challenge with 5 X 10^4 fresh tumor cells.

Fibroblasts mixed with irradiated tumor cells	Animal Number				Tumor Size (mm^2)				Mean Tumor Size (mm^2)
	Total	Tumor-free	Tumor-bearing	Percent Tumor-free	25-100	101-200	201-300	>301	
After 22 Weeks:*									
Control (saline)	20	0	20	0%	0	0	1	19	574 ± 160
Irradiated CT26 only**	16	5	11	31%	2	1	2	5	250 ± 277
Irradiated CT26 mixed with unmodified fibroblasts	15	4	11	27%	0	1	3	7	266 ± 199
DCTK-IL2 fibroblasts**	25	10	15	40%	4	1	1	8	172 ± 194

* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

** One mouse in each of these arms developed an intraperitoneal tumor which was not measurable.

In a second protocol similar to the one described above, animals were challenged with fresh tumor cells two weeks following immunization with irradiated tumor cells mixed with fibroblasts. The results, shown in Figures 14 and 15, and in Table 15, demonstrate that DCTK-IL2 modified fibroblasts mixed with irradiated tumor cells confers superior protection to subsequent tumor challenge than irradiated tumor cells alone, irradiated tumor cells mixed with unmodified fibroblasts, or irradiated tumor cells mixed with LNCX-modified fibroblasts. After 7 weeks, seven of ten animals (70%) treated with DCTK-IL2 modified fibroblasts remain tumor free compared to only one third of the control animals. At four weeks, the mean tumor size of this group was 41 mm^2, compared to 180, 170, and 140 mm^2 for the three control groups. Animals treated with LNCX-IL2 modified fibroblasts were also protected against subsequent tumor challenge, but the results were less striking. In this group, 54% of the animals remain tumor free and the mean tumor size for the group at four weeks was 86 mm^2. The number of tumor free animals in the group treated with LXSN-IL2 modified fibroblasts was similar to the control groups, although the tumors were slightly delayed in their onset. A multivariate non-parametric statistical procedure (19, 20), utilized to evaluate differences in tumor onset, demonstrated that the differences for the six arms presented in Figure 15 were significant ($p = 0.012$). It further showed that the saline control arm and the arms that received irradiated tumor cells alone or mixed with unmodified or LNCX vector modified fibroblasts formed a statistical group. A second, distinct statistical group was formed by the three arms that received IL-2 vector modified fibroblasts mixed with irradiated tumor cells. Subsequent comparisons between the saline injected control arm and animals that received tumor cells mixed with IL2 transduced fibroblasts revealed a significant difference for all vectors ($p < 0.05$).

Table 15

Effect of IL-2 modified fibroblasts on induction of sytemic anti-tumor immunity.
Mice immunized with 2 X 10^6 fibroblasts mixed with 2.5 X 10^5 irradiated CT26 tumor cells 14 days prior to challenge with 5 X 10^4 fresh tumor cells.

Immunization by fibroblasts mixed with irradiated tumor cells	Animal Number Total	Tumor-free	Tumor-bearing	Percent Tumor-free	Tumor Size (mm^2) 25-100	101-200	201-300	>301	Mean Tumor Size (mm^2)
After 7 Weeks:*									
Control (saline)**	8	1	7	13%	0	2	1	3	245 ± 173
Irradiated CT26 only	10	3	7	30%	0	2	4	1	180 ± 155
Irradiated CT26 mixed with unmodified fibroblasts	6	2	4	33%	0	2	1	1	170 ± 160
Irradiated CT26 mixed with LNCX-modified fibroblasts	10	3	7	30%	3	0	1	3	140 ± 142
Irradiated CT26 mixed with LNCX-IL2-modified fibroblasts	13	7	6	54%	1	3	1	1	86 ± 112
Irradiated CT26 mixed with LXSN-IL2-modified fibroblasts	12	4	8	33%	5	0	2	1	111 ± 145
Irradiated CT26 mixed with DCTK-IL2-modified fibroblasts	10	7	3	70%	1	2	0	0	41 ± 75

* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

** One mouse in this arm developed an intraperitoneal tumor which was not measurable.

These results demonstrate the feasibility of using genetically modified fibroblasts as a means of delivering cytokine gene therapy. In all experiments, the LNCX-L2 vector proved superior in preventing tumor establishment while the DCTK-IL2 vector was better in the induction of systemic protection against subsequent tumor challenges. These contrasting effects, although somewhat surprising, can be explained by the observation that the CMV promoter is turned off _in vivo_ five days after implantation while the TK promoter remains active for a longer period of time. The implication of this finding is that to apply this method of gene therapy successfully we have to use promoters that result in high level, sustained expression of IL-2 _in vivo_ in the transduced fibroblasts.

The data obtained from this research effort has important implications for all cytokines that have either direct or indirect anti-tumor effects. Furthermore, this data suggests that anti-tumor efficacy is IL-2 dose dependent. Hence, construction of vectors which result in higher levels of cytokine secretion will be a significant advance toward the application of this method of gene therapy.

Reference numbers in parenthesis in the above examples correspond to the following list of references and are incorporated herein by reference.

References

1. Gabrilove, J.L. et al., Monogr. J. Natl. Cancer Inst. 10:73-7 (1990).

2. Kelso, A., Current Opinion in Immunology, 2:215-25 (1989).

3. Borden, E.C. et al., Cancer, 65:800-14 (1990).

4. Rosenberg, S.A. et al., Ann. Intern. Med., 108:853-864 (1988).

5. Lotze, M.T. et al., JAMA, 256:3117-3124 (1986).

6. Pizza, G. et al., Lymphokine Research, 7:45-8 (1988).

7. Sarna, G. et al., Journal of Biological Response Modifiers, 9:81-6 (1990).

8. Gandolfi, L. et al., Hepato-Gastroenterology, 36:352-6 (1989).

9. Bubenik, J. et al., Immunol. Letters, 19:279-82 (1988).

10. Bubenik et al., Immunol. Letters, 23:287-292 (1990).

11. Fearon, E.R. et al., Cell, 60:387-403 (1990).

12. Gansbacher, B. et al., J. Exp. Med., 172:1217-1224 (1990).

13. Watanabe, Y. et al., Proc. Natl. Acad. Sci., 86:9456-9460 (1989).

14. Tepper, R.I. et al., Cell, 57:503-512 (1989).

15. Kriegler, M., Gene Transfer and Expression: A Laboratory Manual, Stockton Press (1990).

16. Rosenberg, S.A. et al., N. Eng. J. Med., 370 (1990).

17. Cornetta, K. et al., Prog. Nucl. Acid Res. Mol. Biol., 36:311-22 (1989).

18. Hoover, H.C. et al., Cancer Res., 44:1671-76 (1984).

19. Sobol et al. New Eng. J. Med. 316:1111-1117 (1987).

20. Li Xu, et al., Virology, 171:331-341 (1989).

Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

WE CLAIM:

1. A method of treating cancer in a patient comprising the stimulation of that patient's immune response against the cancer by immunizing said patient at a site other than an active tumor site with a formulation comprising tumor antigens and CE cells genetically modified to express at least one cytokine gene product.

2. The method of claim 1 wherein tumor cells previously isolated from said patient provide the tumor antigens.

3. The method of claim 1 wherein the cytokine gene is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, and gamma-interferon.

4. The method of claim 3 wherein one cytokine gene is interleukin-2.

5. The method of claim 1 wherein at least one cytokine gene is transferred into cells to generate CE cells by recombinant methods.

6. The method of claim 5 wherein the cytokine gene is present in an expression vector.

7. The method of claim 6 wherein said expression vector additional contains a suicide gene.

8. The method of claim 5 wherein the CE cells are generated from fibroblasts and antigen-presenting cells.

9. A method for enhancing a patient's immune response to a cancer comprising:

a) isolating fibroblasts from said patient;
b) culturing said fibroblasts *in vitro*;
c) transducing said fibroblasts with a retroviral expression vector containing the gene coding for IL-2 and a gene coding for a tumor antigen in a retroviral expression vector, to express said tumor antigen and to express and secrete said IL-2 by said fibroblasts; and
d) immunizing said patient with said fibroblasts that express IL-2 at a level sufficient to enhance an immune response but low enough to avoid substantial systemic toxicity and that express said tumor antigen, at a site other than an active tumor site.

10. The method of claim 9 wherein said fibroblasts are further modified to express a suicide gene.

11. A composition for increasing a patient's immune response to tumor antigens comprising tumor antigens and CE cells genetically modified to express at least one cytokine gene product.

12. The composition of claim 11 wherein the cytokine gene is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, and gamma interferon.

13. The composition of claim 12 wherein one cytokine gene is interleukin-2.

14. The composition of claim 11 wherein each cytokine gene is expressed at a level sufficient to stimulate the immune response but low enough to avoid substantial systemic toxicities.

15. The method of claim 9 wherein in said transducing step said retroviral expression vector has a promotor causing sustained secretion of IL-2.

16. The method of claim 15 wherein said retroviral expression vector causes the secretion of at least four units of IL-2 per day for a period of ten days or longer.

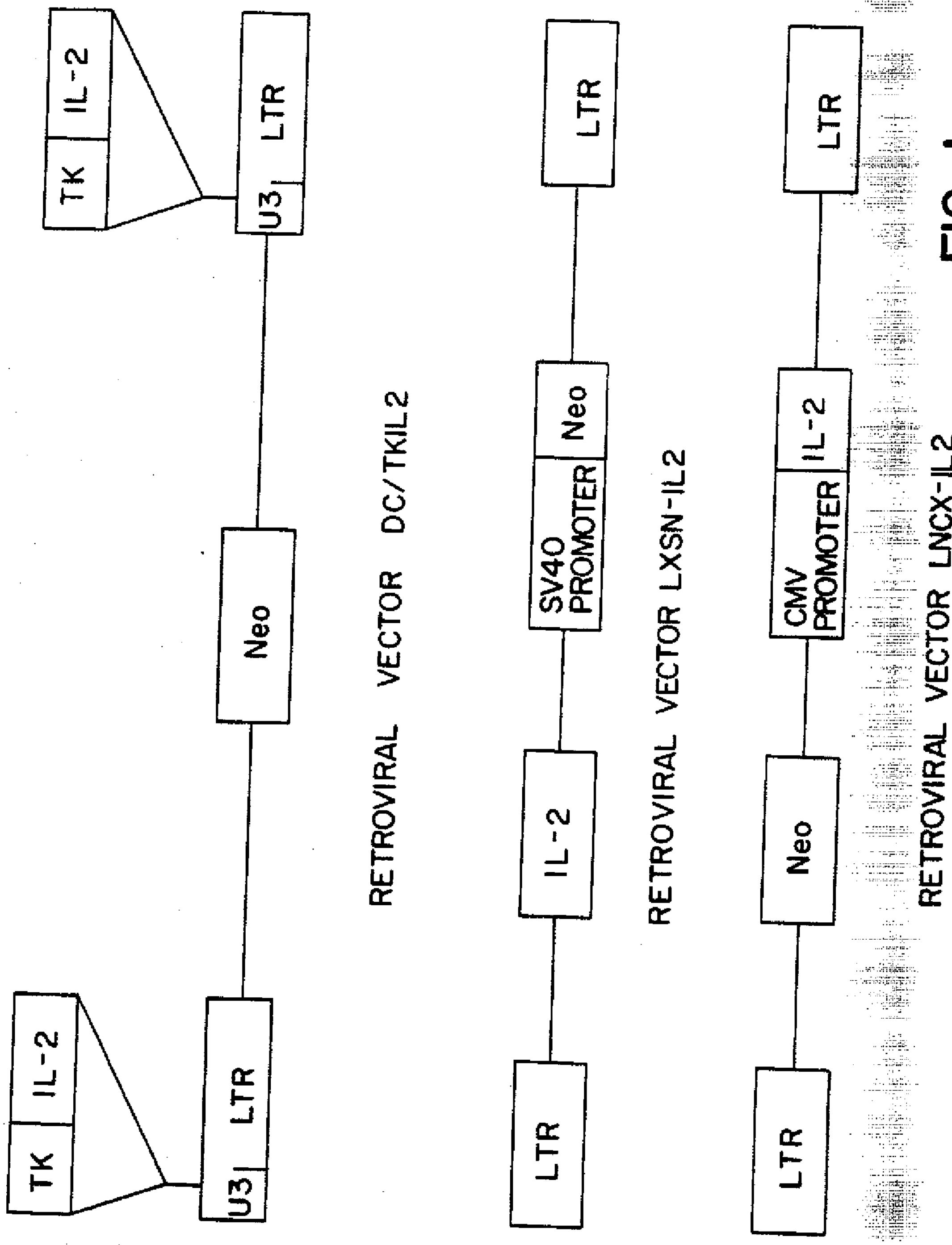

TK
IL-2
U3
LTR
Neo
RETROVIRAL VECTOR DC/TKIL2
LTR
IL-2
SV40 PROMOTER
Neo
RETROVIRAL VECTOR LXSN-IL2
Neo
CMV PROMOTER
RETROVIRAL VECTOR LNCX-IL2

FIG. 1

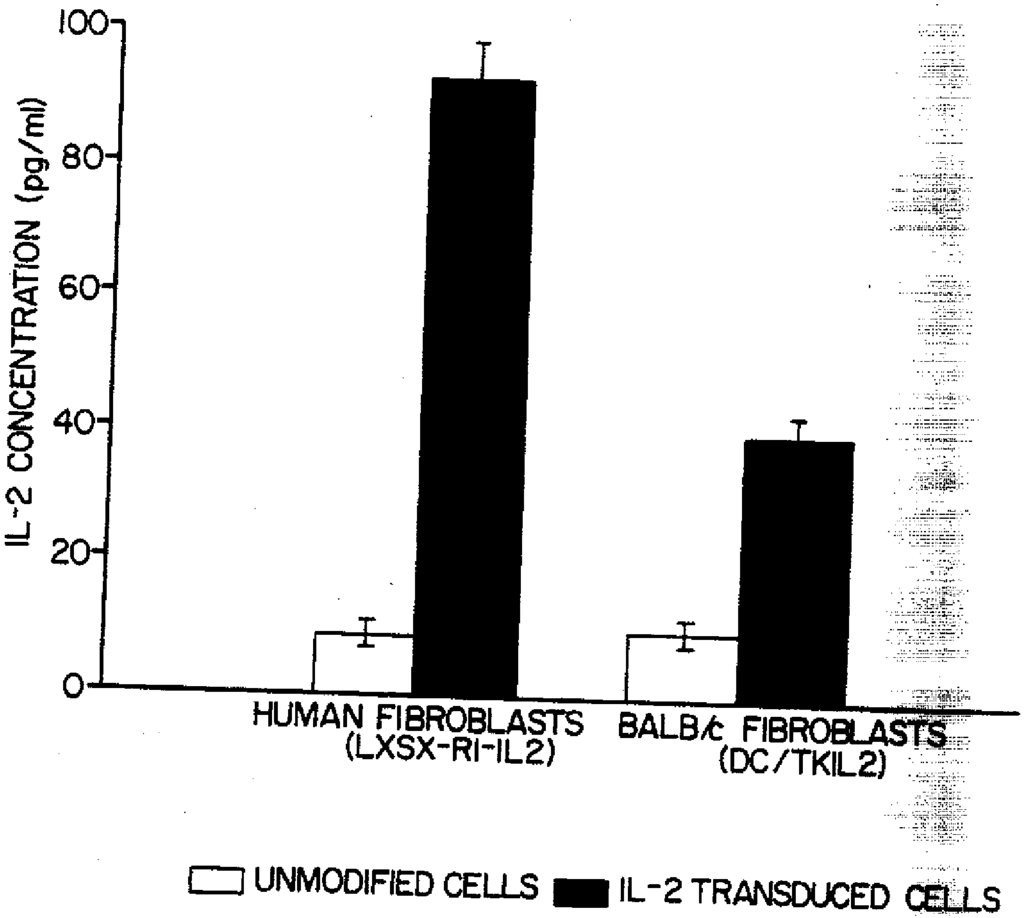
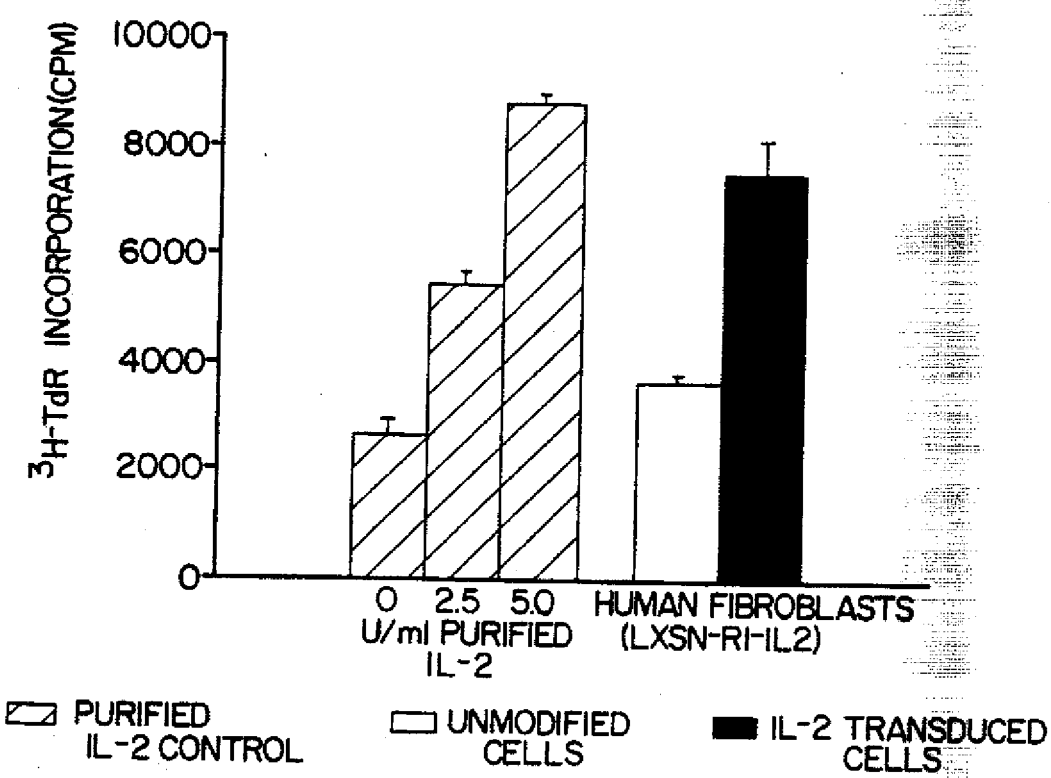

IL-2 CONCENTRATION (pg/ml)
100
80
60
40
20
0
HUMAN FIBROBLASTS
(LXSX-RI-IL2)
BALB/c FIBROBLASTS
(DC/TKIL2)
UNMODIFIED CELLS
IL-2 TRANSDUCED CELLS

FIG. 2



3H-TdR INCORPORATION(CPM)
10000
8000
6000
4000
2000
0
0 2.5 5.0
U/ml PURIFIED
IL-2
HUMAN FIBROBLASTS
(LXSN-RI-IL2)
PURIFIED
IL-2 CONTROL
UNMODIFIED
CELLS
IL-2 TRANSDUCED
CELLS

FIG. 3

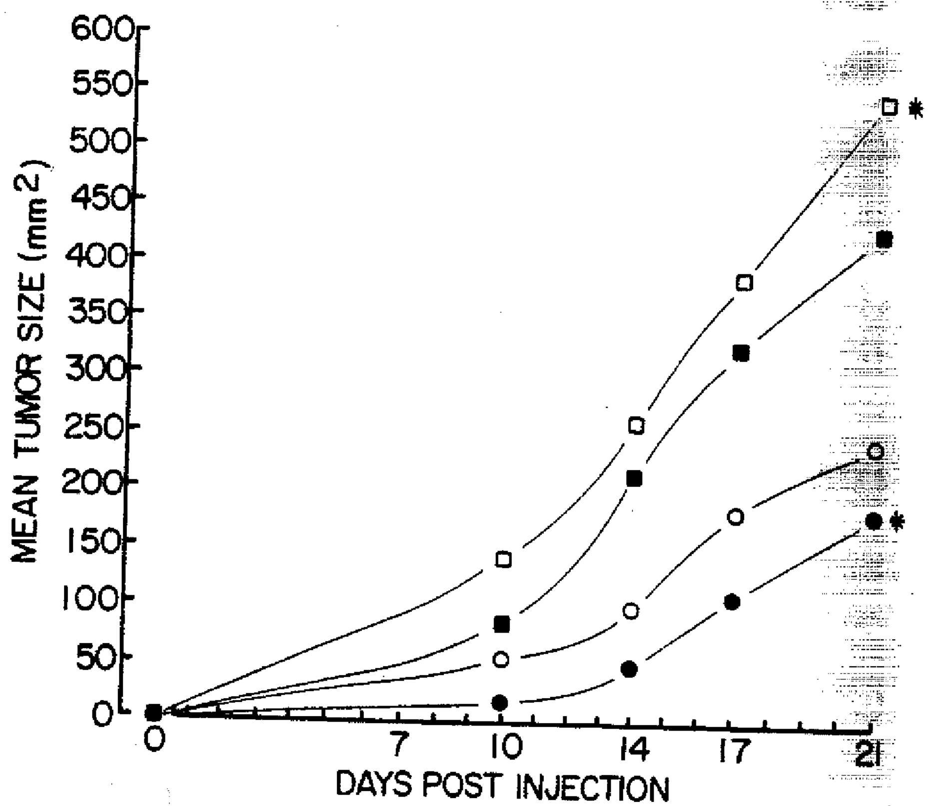

600
550
500
450
400
350
300
250
200
150
100
50
0
MEAN TUMOR SIZE (mm2)
0
7
10
14
17
21
DAYS POST INJECTION
*
*

FIG. 4

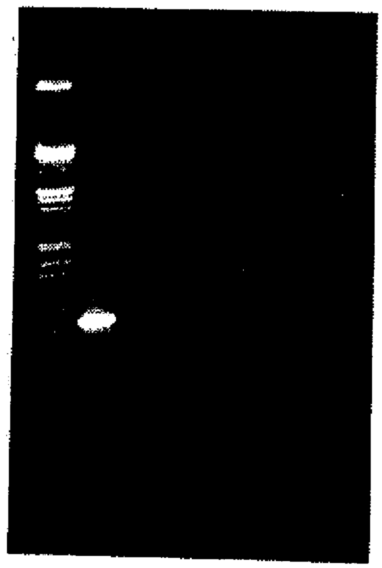
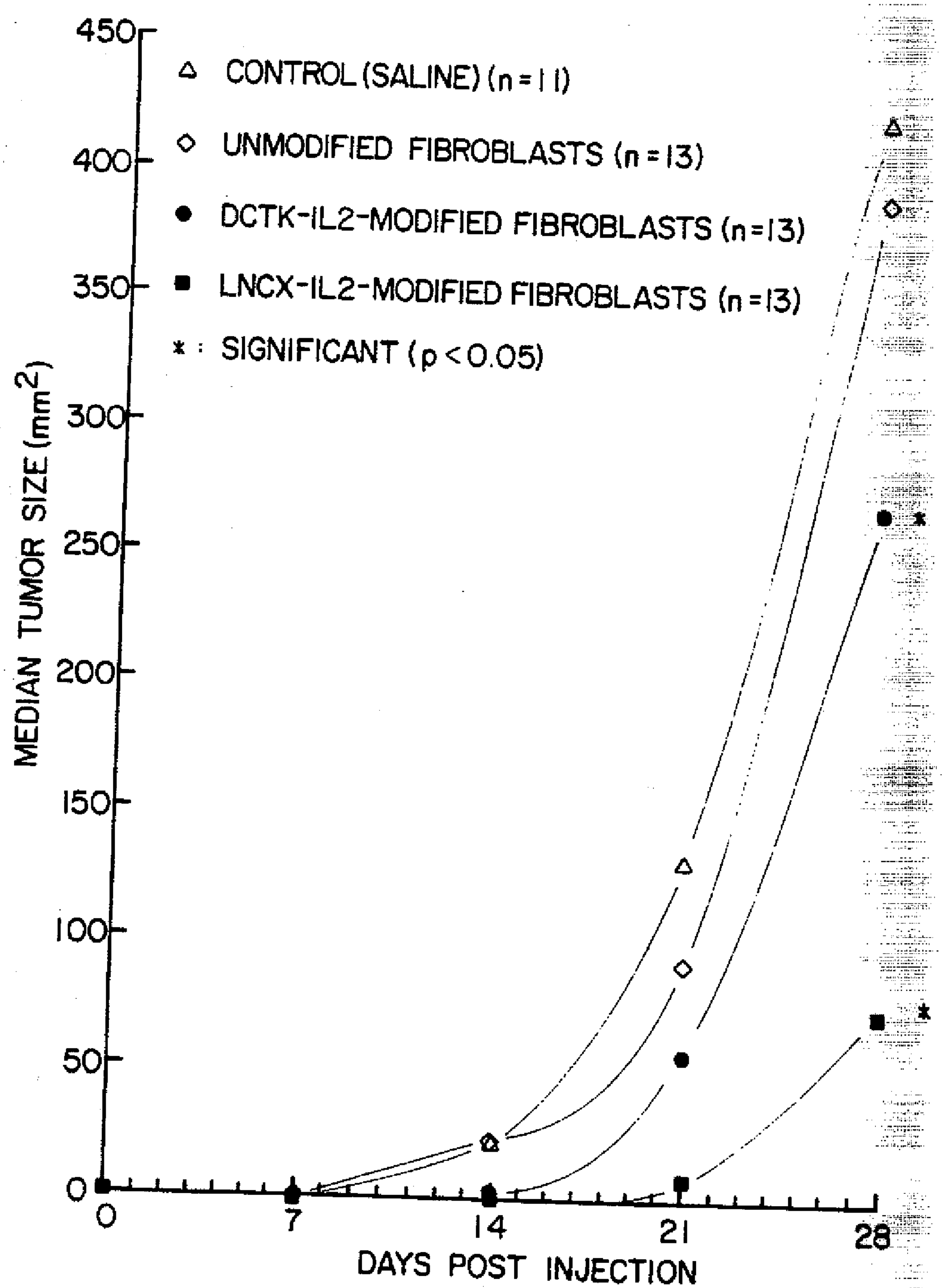
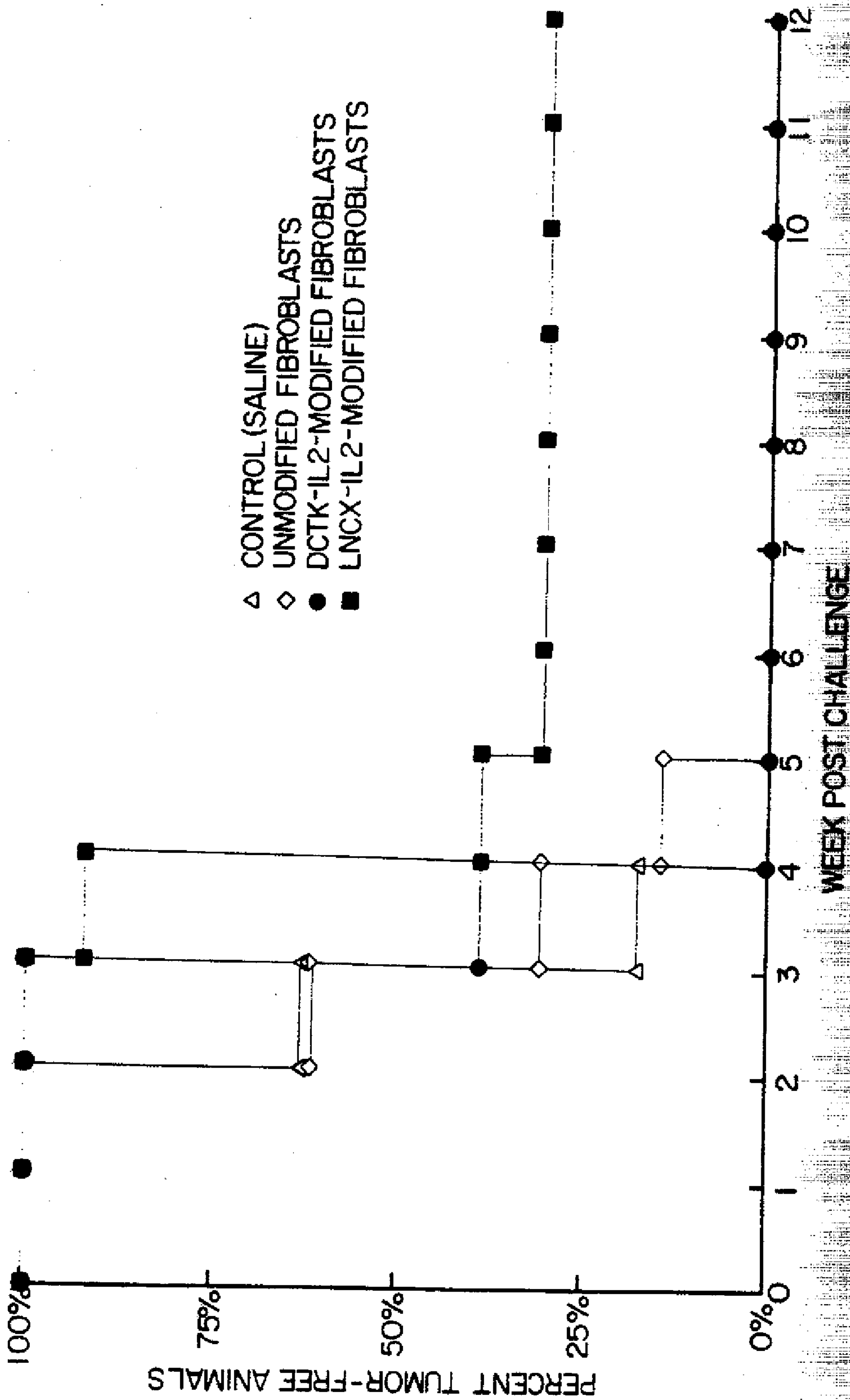


FIG. 5



450
400
350
300
250
200
150
100
50
0
MEDIAN TUMOR SIZE (mm2)
△ CONTROL (SALINE) (n = 11)
◇ UNMODIFIED FIBROBLASTS (n = 13)
● DCTK-IL2-MODIFIED FIBROBLASTS (n = 13)
■ LNCX-IL2-MODIFIED FIBROBLASTS (n = 13)
* : SIGNIFICANT (p < 0.05)
0
7
14
21
28
DAYS POST INJECTION

FIG. 6



CONTROL (SALINE)
UNMODIFIED FIBROBLASTS
DCTK-IL2-MODIFIED FIBROBLASTS
LNCX-IL2-MODIFIED FIBROBLASTS
100%
75%
50%
25%
0%
PERCENT TUMOR-FREE ANIMALS
0
1
2
3
4
5
6
7
8
9
10
11
12
WEEK POST CHALLENGE

FIG. 7

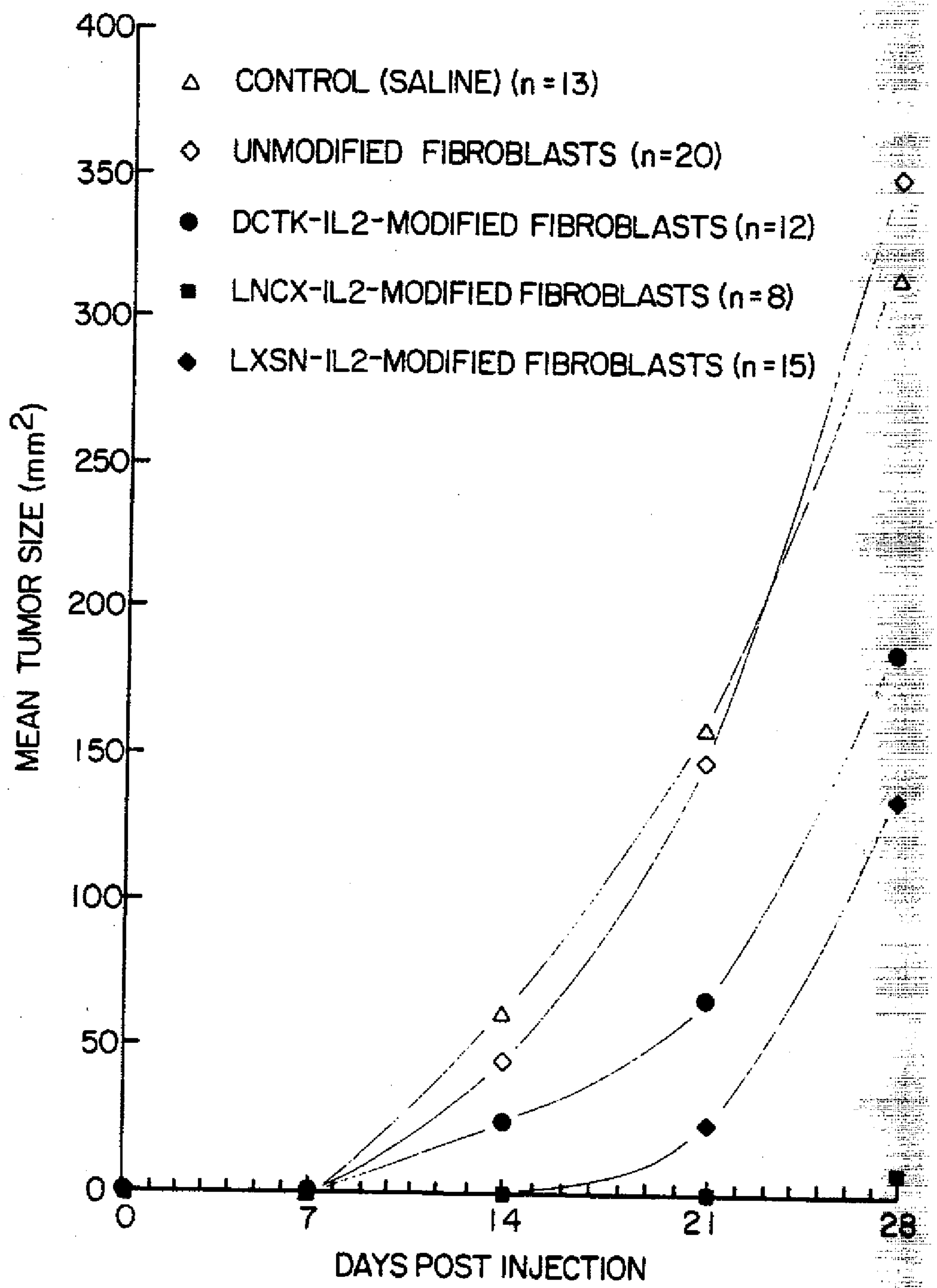

400
350
300
250
200
150
100
50
0
MEAN TUMOR SIZE (mm^2)
CONTROL (SALINE) (n = 13)
UNMODIFIED FIBROBLASTS (n=20)
DCTK-IL2-MODIFIED FIBROBLASTS (n=12)
LNCX-IL2-MODIFIED FIBROBLASTS (n=8)
LXSN-IL2-MODIFIED FIBROBLASTS (n=15)
0
7
14
21
28
DAYS POST INJECTION

FIG. 8

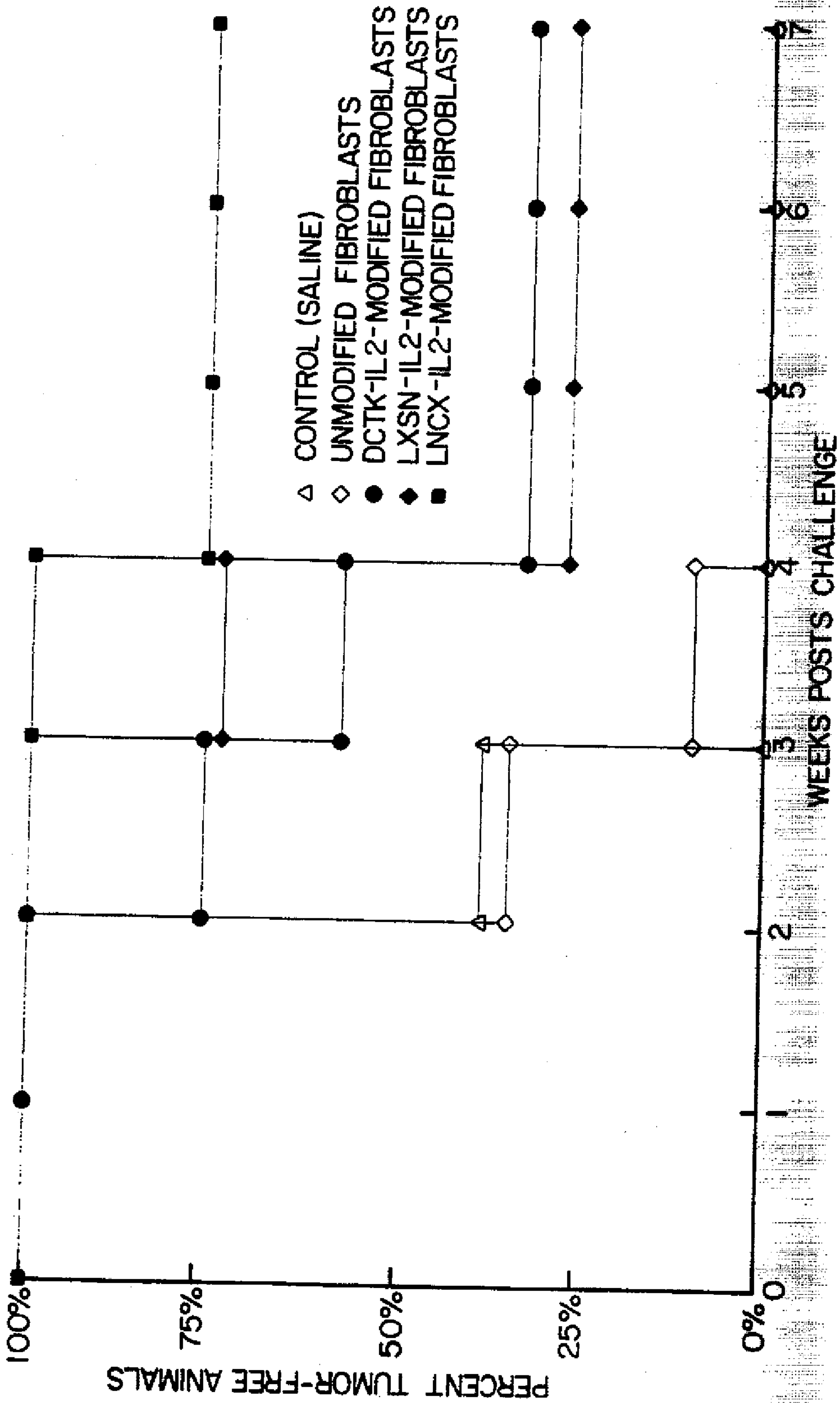

CONTROL (SALINE)
UNMODIFIED FIBROBLASTS
DCTK-IL2-MODIFIED FIBROBLASTS
LXSN-IL2-MODIFIED FIBROBLASTS
LNCX-IL2-MODIFIED FIBROBLASTS
PERCENT TUMOR-FREE ANIMALS
100%
75%
50%
25%
0%
0
1
2
3
4
5
6
7
WEEKS POSTS CHALLENGE

FIG. 9

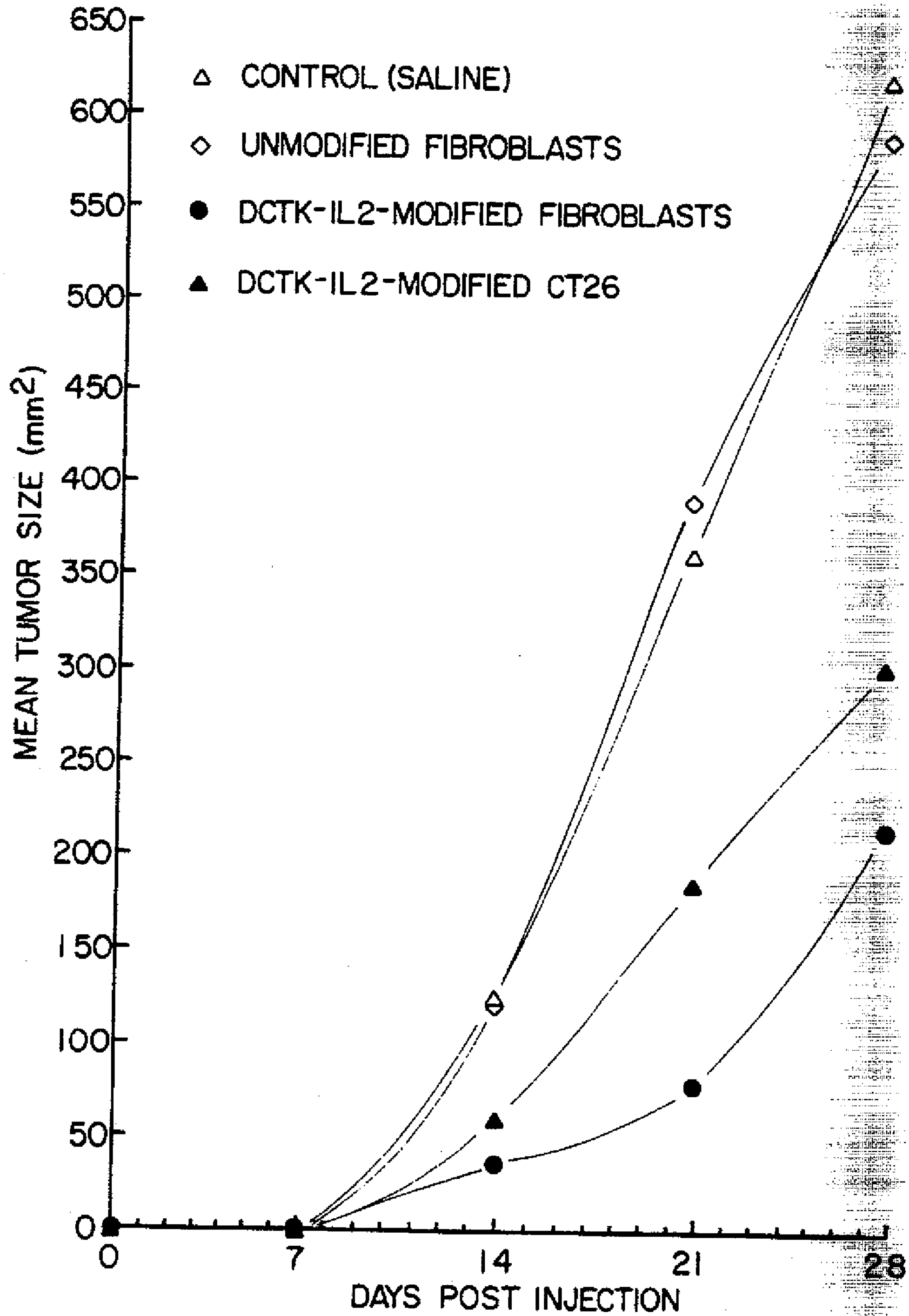

650
600
550
500
450
400
350
300
250
200
150
100
50
0
MEAN TUMOR SIZE (mm^2)
CONTROL (SALINE)
UNMODIFIED FIBROBLASTS
DCTK-IL2-MODIFIED FIBROBLASTS
DCTK-IL2-MODIFIED CT26
0
7
14
21
28
DAYS POST INJECTION

FIG. 10

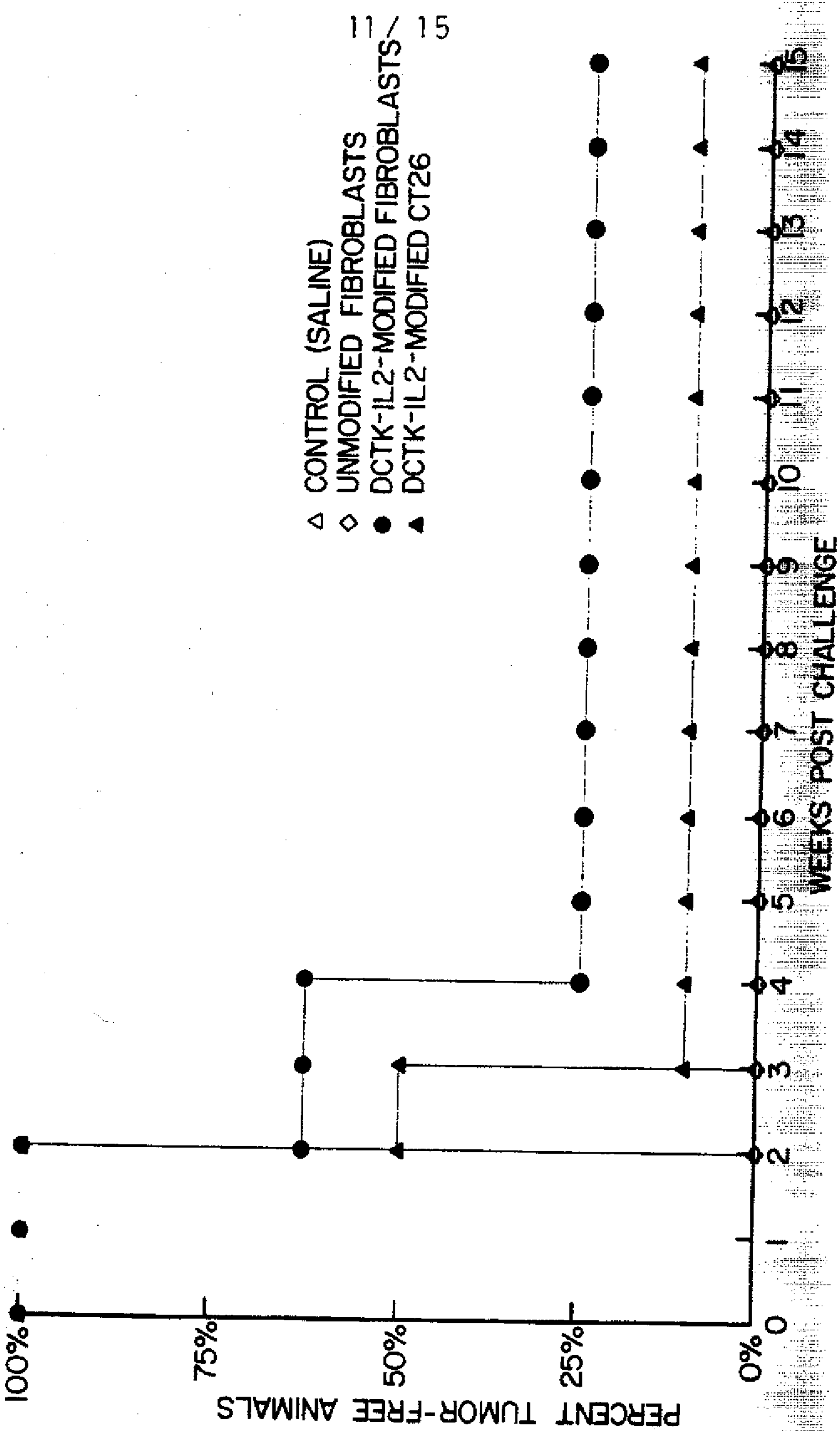
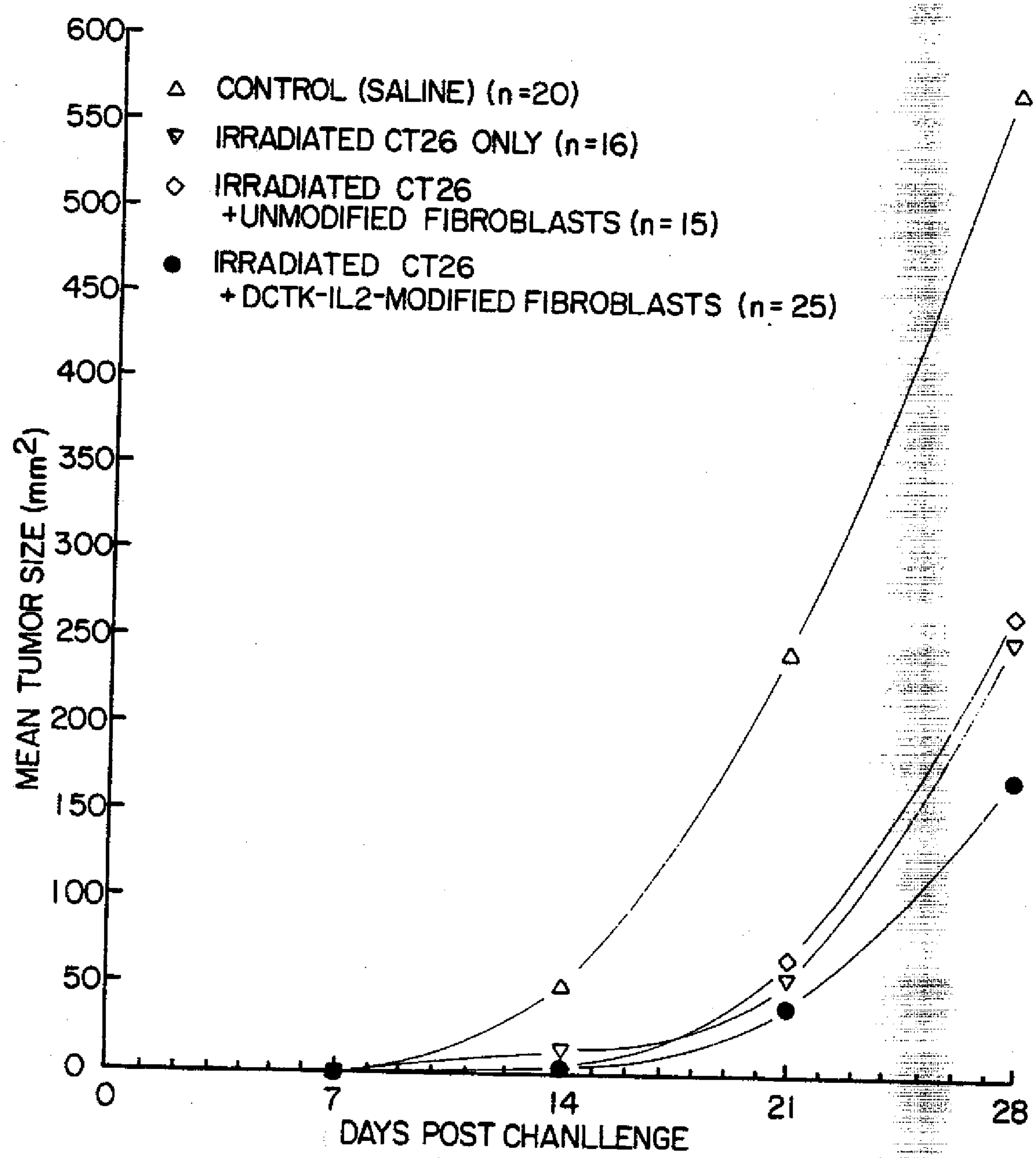

CONTROL (SALINE)
UNMODIFIED FIBROBLASTS
DCTK-IL2-MODIFIED FIBROBLASTS
DCTK-IL2-MODIFIED CT26
PERCENT TUMOR-FREE ANIMALS
100%
75%
50%
25%
0%
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
WEEKS POST CHALLENGE

FIG. 11


600
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500
450
400
350
300
250
200
150
100
50
0
MEAN TUMOR SIZE (mm2)
CONTROL (SALINE) (n=20)
IRRADIATED CT26 ONLY (n=16)
IRRADIATED CT26
+UNMODIFIED FIBROBLASTS (n=15)
IRRADIATED CT26
+DCTK-IL2-MODIFIED FIBROBLASTS (n=25)
0
7
14
21
28
DAYS POST CHANLLENGE

FIG. 12

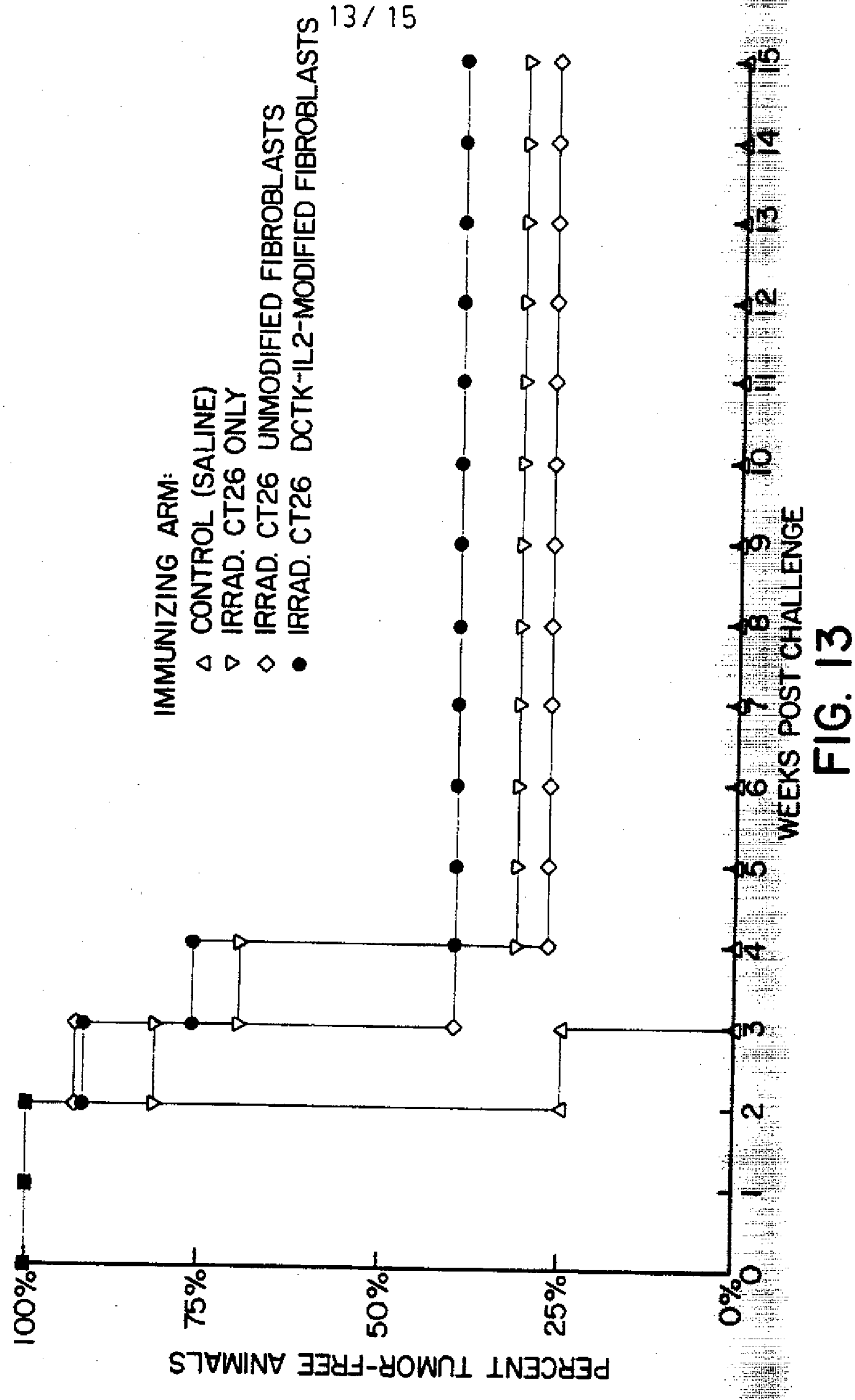
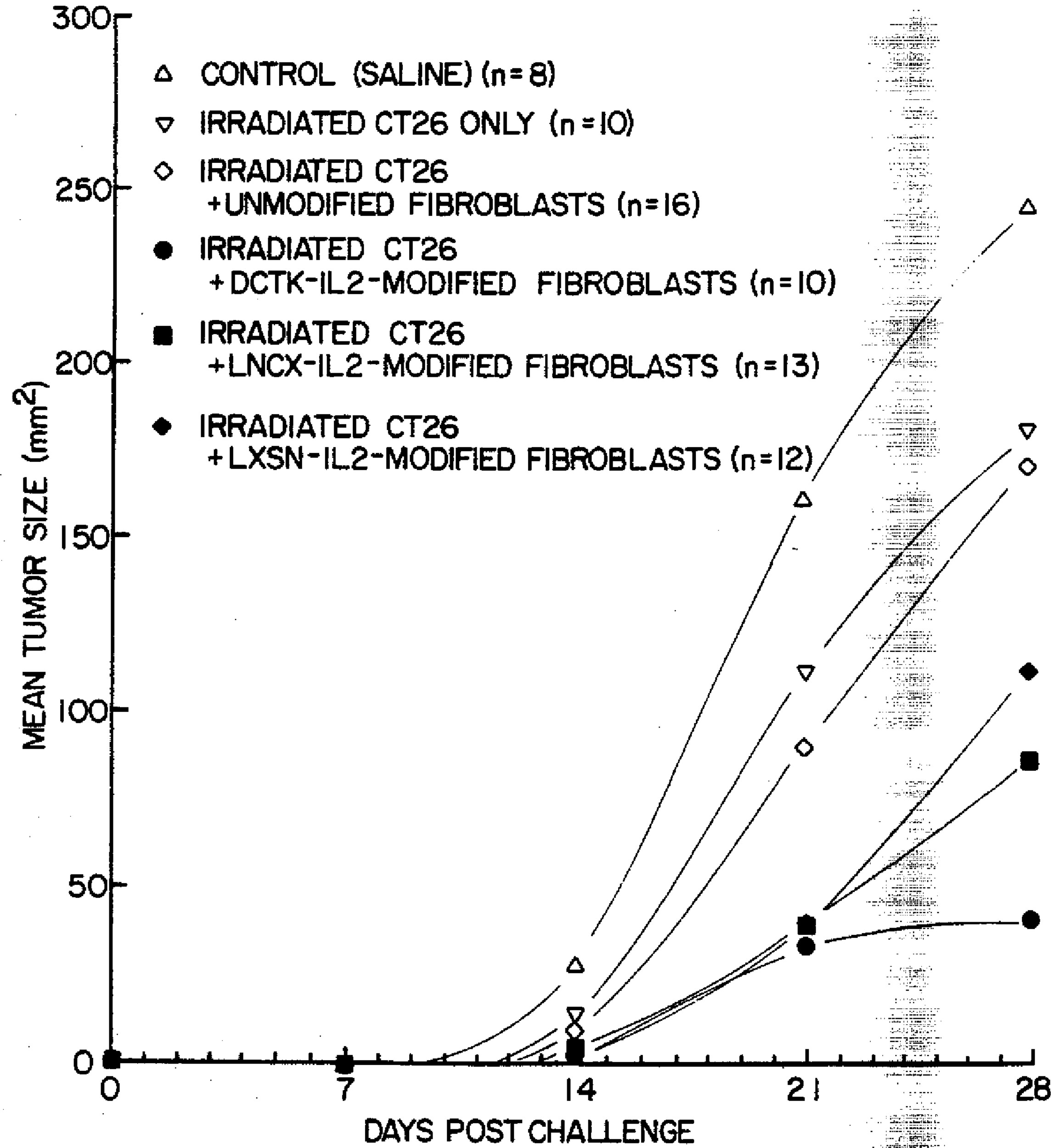

IMMUNIZING ARM:
CONTROL (SALINE)
IRRAD. CT26 ONLY
IRRAD. CT26 UNMODIFIED FIBROBLASTS
IRRAD. CT26 DCTK-IL2-MODIFIED FIBROBLASTS
PERCENT TUMOR-FREE ANIMALS
100%
75%
50%
25%
0%
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
WEEKS POST CHALLENGE

FIG. 13



300
250
200
150
100
50
0
MEAN TUMOR SIZE (mm2)
CONTROL (SALINE) (n=8)
IRRADIATED CT26 ONLY (n=10)
IRRADIATED CT26 +UNMODIFIED FIBROBLASTS (n=16)
IRRADIATED CT26 +DCTK-IL2-MODIFIED FIBROBLASTS (n=10)
IRRADIATED CT26 +LNCX-IL2-MODIFIED FIBROBLASTS (n=13)
IRRADIATED CT26 +LXSN-IL2-MODIFIED FIBROBLASTS (n=12)
0
7
14
21
28
DAYS POST CHALLENGE

FIG. 14

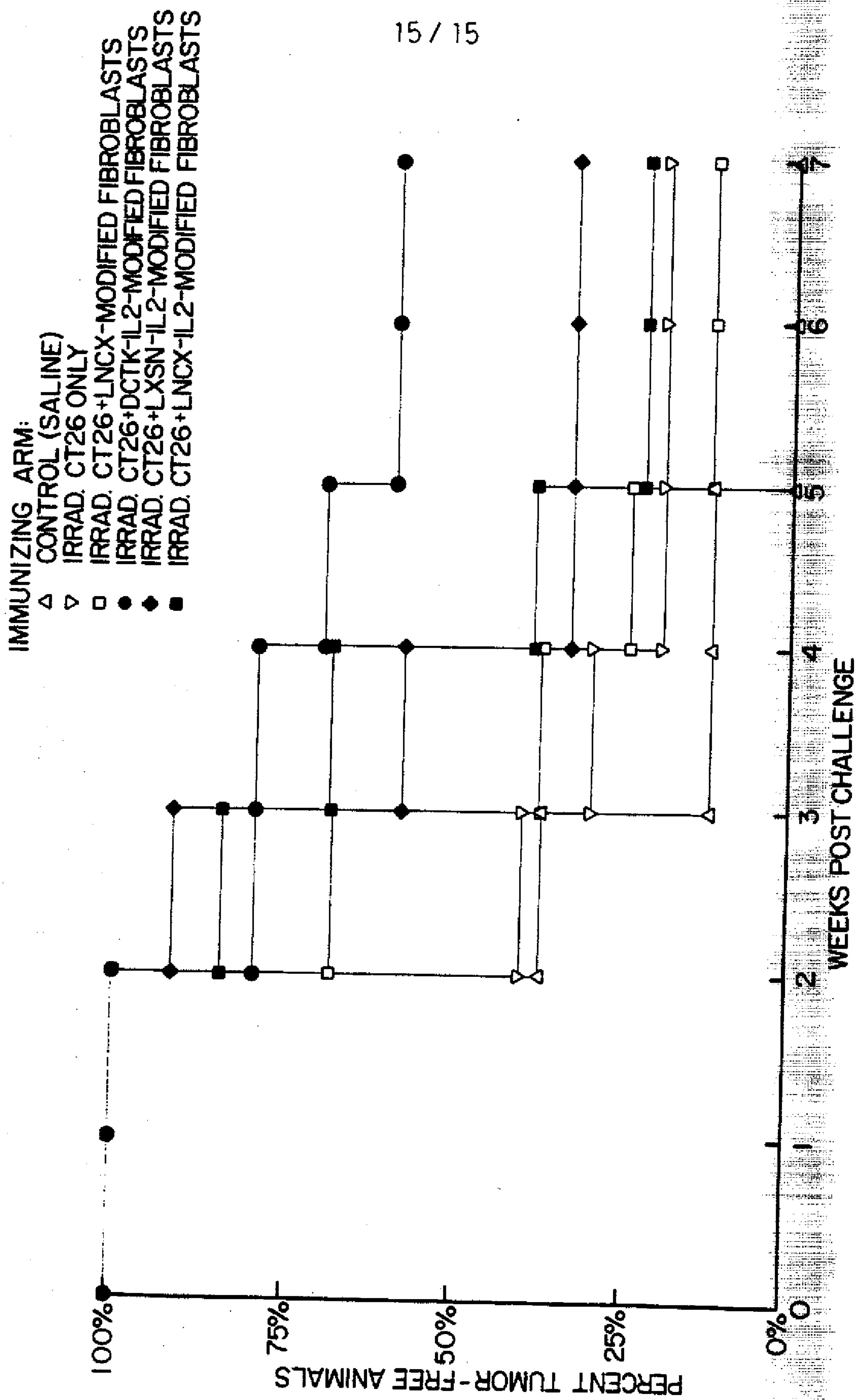

IMMUNIZING ARM:
CONTROL (SALINE)
IRRAD. CT26 ONLY
IRRAD. CT26+LNCX-MODIFIED FIBROBLASTS
IRRAD. CT26+DCTK-IL2-MODIFIED FIBROBLASTS
IRRAD. CT26+LXSN-IL2-MODIFIED FIBROBLASTS
IRRAD. CT26+LNCX-IL2-MODIFIED FIBROBLASTS
100%
75%
50%
25%
0%
PERCENT TUMOR-FREE ANIMALS
0
1
2
3
4
5
6
7
WEEKS POST CHALLENGE

FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08999

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93B, 93U, 89; 435/240.2, 320.1, 69.5, 69.51, 69.52; 935/65, 32, 12, 57, 70, 71

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Journal of Experimental Medicine, Volume 172, issued October 1990, Gansbacher et al, "Interleukin 2 Gene Transfer into Tumor Cells Abrogates Tumorigenicity and Induces Protective Immunity", pages 1217-1224, see the entire document.	1-8, 11-14 9, 10, 15, 16
X Y	Cell, Volume 57, issued 05 May 1989, Tepper et al, "Murine Interleukin-4 Displays Potent Anti-Tumor Activity In Vivo", pages 503-512, see the entire document.	1-3,5,6, 8,11,12,14 4, 13
X Y	Cell, Volume 60, issued 09 February 1990, Fearon et al., "Interleukin-2 Production by Tumor Cells Bypasses T Helper Function in the Generation of an Antitumor Response", pages 397-403, see the entire document.	1,3-5,8, 11-13 2, 6, 7, 14-16
Y	Cancer Research, Volume 50, issued 15 August 1990, Ogura et al., "Implantation of Genetically Manipulated Fibroblasts into Mice as Antitumor α-Interferon Therapy", pages 5102-5106, see the entire document.	1-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 11 January 1993	Date of mailing of the international search report 26 JAN 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JACQUELINE STONE Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Cancer Research, Volume 50, issued 15 December 1990, Gansbacher et al., "Retroviral Vector-mediated Interferon Gene Transfer into Tumor Cells Generates Potent and Long Lasting Antitumor Immunity", pages 7820-7825, see the entire document.	1, 3-5, 6, 8, 11, 12, 14 2,7

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 48/00, 35/12, 39/00; C12N 15/19, 15/24, 15/25, 15/26, 15/90, 15/63

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93B, 93U, 89; 435/240.2, 320.1, 69.5, 69.51, 69.52; 935/65, 32, 12, 57, 70, 71